The roles of deiodinases in thyronamine biology

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Abstract

3-iodothyronamine (3-T_1AM) and thyronamine (T_0AM) are novel endogenous signaling molecules that exhibit great structural similarity to thyroid hormones but apparently antagonize classical thyroid hormone (T_3) actions. The present study investigated whether thyronamines (TAMs) are substrates of the three Dio isozymes (Dio1, Dio2 and Dio3).

TAMs were incubated with isozyme specific Dio preparations. Deiodination products were analyzed using a newly established method applying liquid chromatography and tandem mass spectrometry (LC-MS/MS). Phenolic ring deiodinations of 3,3',5'-triiodothyronamine, 3',5'- and 3,3'-diiodothyronamine as well as tyrosyl ring deiodinations of 3,5,3'-triiodothyronamine and 3,5-diiodothyronamine were observed with preparations containing Dio1. Preparations of Dio2 also deiodinated 3,3',5'-triiodothyronamine and 3',5'-diiodothyronamine at the phenolic rings. All TAMs with tyrosyl ring iodine atoms were deiodinated by Dio3 containing preparations. In functional competition assays, the newly identified TAM substrates inhibited an established iodothyronine deiodination reaction. By contrast, TAMs which had been excluded as Dio substrates in LC-MS/MS experiments, failed to show any effect in the competition assays, thus verifying the former results.

In summary, all three Dio isozymes catalyzed TAM deiodination reactions with each isozyme exhibiting a unique substrate specificity. These data support a role for Dio isozymes in TAM biosynthesis and contribute to confining the biosynthetic pathways of 3-T_1AM and T_0AM. Furthermore, they provide new insights into the structural requirements for Dio substrates in general since TAMs represent the only endogenous Dio substrates described, so far, which possess a positively charged tyrosyl ring side chain.

thyronamine, deiodinase, LC-MS/MS, iodothyronine, thyroid hormone metabolism

Deutscher Abstract

3-Jodthyronamin (3-T_1AM) und Thyronamin (T_0AM) sind endogene Signalmoleküle, die eine große strukturelle Ähnlichkeit zu Schilddrüsenhormonen aufweisen, allerdings die klassischen Wirkungen des aktiven Schilddrüsenhormons 3,5,3'-Trijodthyronin (T_3) antagonisieren. In der vorliegenden Arbeit wurde untersucht, ob Thyronamine (TAMs) Substrate von Dejodasen (Dio1, Dio2, Dio3) sind.

Die TAMs wurden mit isozymspezifischen Dio-Präparationen inkubiert. Die Dejodierungsprodukte wurden mittels Hochleistungsflüssigkeitschromatographie und Tandemmassenspektrometrie (LC-MS/MS) analysiert. Mit Präparationen der Dio1 wurden Dejodierungen von 3,3',5'-Trijodthyronamin, 3',5'- und 3,3'-Dijodthyronamin am phenolischen Ring sowie Dejodierungen von 3,5,3'-Trijodthyronamin und 3,5-Dijodthyronamin am Tyrosylring beobachtet. Dio2 haltige Präparationen katalysierten ebenfalls Dejodierungen von 3,3',5'-Trijodthyronamin und 3',5'-Dijodthyronamin am phenolischen Ring. Mit Dio3 haltigen Präparationen wurden alle TAMs mit jodiertem Tyrosylring dejodiert. In Kompetitionsversuchen inhibierten ausschließlich die TAMs, die als Substrate von Dio Isozymen identifizierten wurden, eine etablierte Dejodierungsreaktion eines bekannten Substrats. Im Gegensatz dazu interferierten TAMs, die in den LC-MS/MS Experimenten als Substrate der Dio Isozyme ausgeschlossen wurden, nicht mit der genannten etablierten Dejodierungsreaktion.

Zusammenfassend wurde in der vorliegenden Arbeit gezeigt, dass TAMs Substrate aller drei Dio Isozyme sind und jedes Isozym eine eigene Substratspezifität aufweist. Diese Befunde weisen darauf hin, dass Dio Isozyme an der Biosynthese von TAMs beteiligt sein könnten. Ferner wurden die Biosynthesewege für 3-T_1AM und T_0AM eingegrenzt. Desweiteren gestatten die Ergebnisse neue Einblicke in die generellen strukturellen Voraussetzungen für Dio Substrate, da TAMs die bisher einzigen endogenen Dio Substrate darstellen, deren Seitenkette am Tyrosylring eine positive Ladung aufweist.

Thyronamin, Dejodase, LC-MS/MS, Jodthyronin, Schilddrüsenhormonmetabolismus

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Berlin, 2008

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Dedicated to my mom.

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°C	degree Celsius	**DNA**	**d**eoxyribo**n**ucleic **a**cid
3-T_1AMS	**s**ulfated L-3-iodo**t**hyron**am**ine	**dNTP**	**d**eoxyribo**n**ucleoside-**t**ri**p**hosphate
Å	angstrom	**DP**	**d**eclustering **p**otential
AADC	**a**romatic **a**mino acid **d**e**c**arboxylase	**dsDNA**	**d**ouble **s**trand **d**eoxyribo**n**ucleic **a**cid
amu	**a**tomic **m**ass **u**nit	**DTE**	**di**thio**e**rythritol
API	**a**tmospheric **p**ressure **i**onization	**DTT**	DL-**di**thio**t**hreitol
ATP	**a**denosine **t**ri**p**hosphate	**dTTP**	**d**eoxy**t**hymidine-5'-**t**ri**p**hosphate
AUC	**a**rea **u**nder the **c**urve	**DUOX**	**du**al **ox**idase
bp	**b**ase **p**air(s)	**e.g.**	for example
BSA	**b**ovine **s**erum **a**lbumin	**EC_{50}**	half maximal **e**ffective **c**oncentration
C57BL/6	inbred mouse strain	**EDTA**	**e**thylene**d**iamine**t**etra**a**cetic acid
CAD	**c**ollision **a**ctivated **d**issociation	**EP**	**e**ntrance **p**otential
cAMP	**c**yclic **a**denosine **m**ono**p**hosphate	**ESI (+)**	positive **e**lectrospray **i**onization
cDNA	**c**omplementary **DNA**	**FCS**	**f**etal **c**alf **s**erum
CE	**c**ollision **e**nergy	**FP**	**f**ocussing **p**otential
CEP	**c**ell **e**ntrance **p**otential	**FSH**	**f**ollicle **s**timulating **h**ormone
cpm	**c**ounts **p**er **m**inute	**fwd**	**f**or**w**ar**d**
cps	**c**ounts **p**er **s**econd	**g**	**g**ram
CUR	**cu**rtain **g**as	**GAPDH**	**g**lycerin**a**ldehyde-3-**p**hosphate **de**h**h**ydrogenase
CV	**c**oefficient of **v**ariation	**GPCR**	**G**-**p**rotein **c**oupled **r**eceptor
D	**D**alton	**h**	**h**our
dATP	**d**eoxy**a**denosine-5'-**t**ri**p**hosphate	**hCG**	**h**uman **c**horionic **g**onadotropin
dCTP	**d**eoxy**c**ytidine-5'-**t**ri**p**hosphate	**HEPES**	4-(2-**h**ydroxy**e**thyl)**p**iperazine-1-**e**thane**s**ulfonic acid
ddH_2O	**d**ouble **d**istilled water	**His**	**his**tidine
DEPC	**die**thyl**p**yro**c**arbonate	**HPLC**	**h**igh **p**erformance **l**iquid **c**hromatography
dGTP	**d**eoxy**g**uanosine-5'-**t**ri**p**hosphate	**i.e.**	that is
Dio	**d**e**io**dinase	**IgG**	**i**mmuno**g**lobulin **G**
DIT	**di**iodo-**t**yrosyl residue	**IS**	**i**nternal **s**tandard, here 3-T_1AM-d4
DMEM	**D**ulbecco's **M**odified **E**agle **M**edium	**K_i**	dissociation constant
DMSO	**dim**ethyl**s**ulf**o**xide	**K_m**	Michaelis-Menten constant

l	**l**iter
L	levorotatory
LC-MS(/MS)	**l**iquid **c**hromatography (tandem) **m**ass **s**pectometry
LH	**l**uteinizing **h**ormone
LOD	**l**imit **o**f **d**etection
LOQ	**l**imit **o**f **q**uantification
M	**m**olar
m	**m**eter
m/z	mass-to-charge ratio
MAPK	**m**itogen **a**ctivated **p**rotein **k**inase
MCT	**m**ono**c**arboxylate **t**ransporter
min	minute
MIT	**m**ono**i**odo-**t**yrosyl residue
mol	mole
MOPS	3-(N-**mo**rpholino)**p**ropane**s**ulfonic acid
mRNA	**m**essenger **r**ibo**n**ucleic **a**cid
na	**n**ot **a**pplicable
$Na_2EDTA•H_2O$	**e**thylene**d**iamine**t**etra**a**cetic acid disodium salt dihydrate
nd	**n**ot **d**etectable
NEB	**neb**ulizer gas
NIS	**N**a^+/**I** $^-$ **s**ymporter
OATP	**o**rganic **a**nion **t**ransporting **p**oly-peptide
OD	**o**ptical **d**ensity
PBS	**p**hosphate **b**uffered **s**aline
PCR	**p**olymerase **c**hain **r**eaction
PI3K	**p**hospho**i**nositide **3**-**k**inase
PMA	**p**horbol-12-**m**yristate-13-**a**cetate
$Pro\text{-}NH_2$	amidated proline
PTU	6-n-**p**ropyl-2-**t**hio**u**racil
PTX	*Bordetella **p**ertussis* to**x**in
pyro-Glu	pyroglutamate
Q1	first quadrupol of tandem mass spectrometer
Q3	third quadrupol of tandem mass spectrometer
r^2	coefficient of determination
rev	**rev**erse
RIA	**r**adio**i**mmuno **a**ssay
RNA	**r**ibo**n**ucleic **a**cid
RP	**r**eversed **p**hase
RPMI-1640	**R**oswell **P**ark **M**emorial **I**nstitute, cell culture medium
RQ	**r**espiratory **q**uotient
rRNA	**r**ibosomal **r**ibo**n**ucleic **a**cid
rt	**r**etention **t**ime
RT	**r**everse **t**ranscription
rT_3	**r**everse (3,3',5'-) triiodo**t**hyronine
rT_3AM	**r**everse (3,3',5'-) triiodo**t**hy-ron**am**ine
RT-PCR	**r**everse **t**ranscription-**p**olymerase **c**hain **r**eaction
RXR	9-*cis*-**r**etinoic-**X**-**r**eceptor
s	**s**econd
S	**s**vedberg
SD	**s**tandard **d**eviation
Sec	**se**leno**c**ysteine
SECIS	**se**leno**c**ystein **i**nsertion **s**equence
SRM	**s**elected **r**eaction **m**onitoring
SULT	**sul**fo**t**ransferase
T_0	L-**t**hyronine
T_0AM	L-**t**hyron**am**ine
T_0AMS	**s**ulfated L-**t**hyron**am**ine
T_1	L-monoiodo**t**hyronine
T_1AM	L-monoiodo**t**hyron**am**ine
T_2	L-diiodo**t**hyronine

T_2AM	L-diiodo**t**hyron**am**ine	**TR**	**t**hyroid hormone **r**eceptor
T_3	L-3,5,3'-triiodo**t**hyronine	**TRE**	**t**hyroid hormone **r**esponse **e**lement
T_3AM	L-3,5,3'-triiodo**t**hyron**am**ine	**TRH**	**T**SH **r**eleasing **h**ormone
T_3AMS	sulfated L-3,5,3'- triiodo**t**hy-ron**am**ine	**triac**	**tri**iodothyro**ac**etic acid
T_4	L-3,5,3',5'-tetraiodo**t**hyronine	**tris**	**tris**(hydroxymethyl)aminomethane
T_4AM	L-3,5,3',5'-tetraiodo**t**hyron**am**ine	**tRNA**	**t**ransfer **r**ibo**n**ucleic **a**cid
TAAR	**t**race **a**mine **a**ssociated **r**eceptor	**$tRNA^{Ser(Sec)}$**	Sec-specific tRNA
TAE	**t**ris-**a**cetate-**E**DTA	**TSH**	**t**hyroid **s**timulating **h**ormone
TAM(s)	refers to all **t**hyron**am**ines	**TTR**	**t**rans**t**hy**r**etin
TBA	**t**hyroxine **b**inding **a**lbumin	**UV/Vis**	**u**ltraviolet-**v**isible
TBG	**t**hyroxine **b**inding **g**lobulin	**V**	**v**olt
tetrac	**tetr**aiodothyro**ac**etic acid	**v_{max}**	**max**imum reaction velocity
Tg	**t**hyro**g**lobulin	**w**	**w**att
TH(s)	refers to all **t**hyronines	**w/o**	**w**ith**o**ut
TPO	**t**hyroid **p**er**o**xidase	**x g**	gravity

Summary

Thyronamines (TAMs) are a novel class of endogenous signaling compounds. In terms of structure, they differ from thyronines (THs), such as the thyroid hormone L-thyroxine (T_4) and deiodinated thyroid hormone derivatives, merely concerning the absence of the carboxylate group of the β-alanine side chain. So far, only two representatives of TAMs, namely 3-iodothyronamine (3-T_1AM) and thyronamine (T_0AM), have been detected *in-vivo* in various species. Although their physiological roles still remain elusive, 3-T_1AM and T_0AM have exhibited short-term hypothermic, negative chronotropic and negative inotropic effects that are opposite in direction to the actions of the classical biologically active thyroid hormone 3,5,3'-triiodothyronine (T_3). Thus, TAMs were suggested to be derivatives of thyroid hormones that might serve to fine-tune or even antagonize thyroid hormone effects.

Yet, the pathways of TAM biosynthesis are still unknown. If TAMs were derivatives of THs (e.g. T_4 or T_3), a decarboxylation of the β-alanine side chain would be required for their biosynthesis. However, so far, no TH decarboxylating enzyme has been identified. If the putative decarboxylating enzyme was converting only THs with higher iodine content, deiodinases (Dio) would be directly required to complete 3-T_1AM and T_0AM biosynthesis by removing one to four iodine atoms. A role of Dio isozymes in TAM biosynthesis presupposes their ability to accept TAMs as substrates.

So far, the deiodinase isozymes (Dio1, Dio2 and Dio3) have been described to catalyze the sequential reductive removal of iodine from THs and various TH metabolites thus controling the bioavailability of thyroid hormones. While Dio1 exhibits both phenolic and tyrosyl ring deiodination activity, Dio2 and Dio3 are more specific with respect to the position of the iodine atom removed. Dio2 catalyzes only deiodination reactions of the phenolic ring, e.g. the conversion of the prohormone T_4 to active T_3, whereas Dio3 catalyzes only deiodination reactions of

the tyrosyl ring, e.g. the conversion of T_4 to inactive 3,3',5'-triiodothyronine (reverse T_3, rT_3).

In the present study the complete panel of TAM deiodination reactions was investigated systematically. Since the various TAMs differ only regarding the number or the position of the iodine atoms, their distinction by immunological methods has been hampered, so far. Therefore, a novel liquid chromatography tandem mass spectrometry (LC-MS/MS) method was developed, which allowed for the simultaneous detection of all TAMs in the same sample. TAMs were incubated with isozyme specific Dio preparations. The deiodination products were analyzed using LC-MS/MS. Phenolic ring deiodinations of 3,3',5'-triiodothyronamine (rT_3AM), 3',5'-diiodothyronamine (3',5'-T_2AM), 3,3'-diiodothyronamine (3,3'-T_2AM) as well as tyrosyl ring deiodinations of 3,5,3'-triiodothyronamine (T_3AM) and 3,5-diiodothyronamine (3,5-T_2AM) were observed with Dio1. Dio2 containing preparations also deiodinated rT_3AM and 3',5'-T_2AM at the phenolic rings. All TAMs with tyrosyl ring iodine atoms were deiodinated by Dio3 containing preparations. In functional competition assays, the newly identified TAM substrates inhibited an established iodothyronine deiodination reaction. By contrast, TAMs which had been excluded as Dio substrates in LC-MS/MS experiments, failed to show any effect in the competition assays, thus verifying the former results. Finally, the apparent K_m and v_{max} of selected TAM deiodination reactions were studied and compared to that of the corresponding TH. Some TAMs displayed a higher v_{max}/K_m ratios and thus represented better substrates of the respective Dio isozyme than their corresponding TH.

In summary, all three Dio isozymes catalyzed TAM deiodination reactions with each isozyme exhibiting a unique substrate specificity. These data support a role for Dio isozymes in TAM biosynthesis and contribute to confining the biosynthetic pathways for 3-T_1AM and T_0AM. Moreover, they provide new insights into the structural requirements of Dio substrates in general since TAMs represent the only endogenous Dio substrates described, so far, which possess a positively charged tyrosyl ring side chain.

Zusammenfassung

Thyronamine (TAMs) sind eine neue Gruppe endogener Signalmoleküle. Sie unterscheiden sich strukturell von Thyroninen (THs), wie zum Beispiel dem Schilddrüsenprohormon Thyroxin (T_4) und dessen Dejodierungsprodukten, nur durch das Fehlen der Carboxylgruppe in der β-Alanin-Seitenkette. Bisher wurden zwei Vertreter der TAMs, genauer 3-Jodthyronamin (3-T_1AM) und Thyronamin (T_0AM), *in-vivo* in verschiedenen Spezies nachgewiesen. Die physiologische Funktion der TAMs ist nicht bekannt. 3-T_1AM und T_0AM führten in Tierversuchen in pharmakologischen Dosen zu einem schnellen Abfall der Körpertemperatur um fast 8 °C sowie zu negativer Chronotropie und Inotropie. Damit lösen TAMs Effekte aus, die den klassischen Wirkungen des biologisch aktiven Schilddüsenhormons 3,5,3'-Trijodthyronin (T_3) direkt entgegenstehen.

Aufgrund der gegensätzlichen Wirkungen von TAMs und Schilddrüsenhormonen wurde mehrfach die Hypothese formuliert, TAMs wären Metabolite von Schilddrüsenhormonen und könnten deren Wirkung antagonisieren. Die Biosynthesereaktionen für TAMs sind allerdings nicht bekannt. Falls TAMs als Metabolite von THs, zum Beispiel T_4 und T_3, biosynthetisiert werden, müsste deren β-Alanin-Seitenkette decarboxyliert werden. Bisher wurde allerdings noch keine TH spezifische Decarboxlaseaktivität beschrieben. Falls diese putative Decarboxylase nur THs mit höherem Jodgehalt umsetzt, wären Dejodasen (Dio) notwendig, um die Biosynthese von 3-T_1AM und T_0AM durch die Abspaltung von einem bis vier Jodatomen abzuschließen. Eine Funktion der Dio Isozyme bei der Biosynthese der TAMs setzt voraus, dass TAMs dejodiert werden.

Bisher wurde beschrieben, dass drei Dio Isozyme (Dio1, Dio2 und Dio3) die reduktive Abspaltung von Jodatomen aus THs und verschiedenen TH-Metaboliten katalysieren und damit die Bioverfügbarkeit der Schilddrüsenhormone regulieren. Während Dio1 sowohl Dejodierungen am phenolischen und am Tyrosylring katalysiert, haben Dio2 und Dio3 begrenztere katalytische Aktivitäten. Dio2 katalysiert ausschließlich Dejodierungen am phenolischen Ring, zum Beispiel die Synthese

des aktiven T_3 aus dem Prohormon T_4. Dio3 hingegen katalysiert ausschließlich Dejodierungen am Tyrosylring, zum Beispiel den Abbau von T_4 zum inaktiven 3,3',5'-Trijodthyronin (reverses T_3, rT_3).

In der vorliegenden Arbeit wurden alle TAM Dejodierungsreaktionen systematisch anaylsiert. Da sich die TAMs untereinander nur hinsichtlich der Anzahl oder Position der Jodatome unterscheiden, wurde in dieser Arbeit eine auf Hochleistungsflüssigkeitschromatographie und Tandemmassenspektrometrie (LC-MS/MS) basierende Methode entwickelt, die es ermöglichte, alle TAMs in einer Probe in einem Analysenlauf zu detektieren. Die TAMs wurden mit isozymspezifischen Dio-Präparationen inkubiert und die Dejodierungsprodukte mittels LC-MS/MS analysiert. Mit Präparationen der Dio1 wurden Dejodierungen von 3,3',5'-Trijodthyronamin (rT_3AM), 3',5'-Dijodthyronamin (3',5'-T_2AM) und 3,3'-Dijodthyronamin (3,3'-T_2AM) am phenolischen Ring sowie Dejodierungen von 3,5,3'-Trijodthyronamin (T_3AM) und 3,5-Dijodthyronamin (3,5-T_2AM) am Tyrosylring beobachtet. Dio2 haltige Präparationen katalysierten ebenfalls Dejodierungen von rT_3AM und 3',5'-T_2AM am phenolischen Ring. Mit Dio3 haltigen Präparationen wurden alle TAMs mit jodiertem Tyrosylring auch entsprechend am Tyrosylring dejodiert. In Kompetitionsversuchen inhibierten ausschließlich die TAMs, die als Substrate von Dio Isozymen identifizierten wurden, eine etablierte Dejodierungsreaktion eines bekannten Substrats. Im Gegensatz dazu interferierten TAMs, die in den LC-MS/MS Experimenten als Substrate der Dio Isozyme ausgeschlossen wurden, nicht mit der genannten etablierten Dejodierungsreaktion. Mit diesen Befunden wurden die Ergebnisse LC-MS/MS-Experimente bestätigt. Schließlich wurden die apparenten K_m- und v_{max}-Werte repräsentativer TAM-Dejodierungsreaktionen gemessen und mit denen des korrespondieren THs verglichen. Dabei wurde in einigen Fällen das TAM mit einem höheren apparenten v_{max}/K_m Verhältnis umgesetzt und stellte somit ein besseres Dio-Substrat dar, als das entsprechende TH.

Zusammenfassend wurde in der vorliegenden Arbeit gezeigt, dass TAMs Substrate aller drei Dio Isozyme sind. Jedes Isozym wies eine eigene Substratspezifi-

tät auf. Die Befunde dieser Arbeit weisen darauf hin, dass Dio Isozyme an der Biosynthese von TAMs beteiligt sein könnten. Ferner wurden auf dieser Grundlage die Biosynthesewege für 3-T_1AM und T_0AM eingegrenzt. Desweiteren gestatten die Ergebnisse neue Einblicke in die generellen strukturellen Voraussetzungen für Dio Substrate, da TAMs die bisher einzigen endogenen Dio Substrate darstellen, deren Seitenkette am Tyrosylring eine positive Ladung aufweist. .

1 Introduction

1.1 Thyronines

1.1.1 Thyroidal biosynthesis of T_4 and T_3

The major secretion product of the thyroid gland is L-3,5,3',5'-tetraiodothyronine, which is also referred to as thyroxine or T_4 [Engler, 84]. T_4 is biosynthesized in a multistep process as an iodinated derivative of the amino acid tyrosine (Figure 1A). To concentrate iodine within the thyroid gland, the Na^+/I^- symporter (NIS) localized to the basolateral plasma membrane of the thyrocytes actively transports iodide from the blood stream into the thyrocyte [Dai, 96; Smanik, 96; Riesco-Eizaguirre, 06]. The trapped iodide is further transported across the apical plasma membrane into the follicular lumen by the pendrin transporter [Royaux, 00]. The follicular lumen contains colloid, which mainly consists of thyroglobulin (Tg), a 660 kD glycoprotein rich in tyrosine and secreted from thyrocytes [Taurog, 00]. Several steps of T_4 biosynthesis are catalyzed by the thyroid peroxidase (TPO), which is a hemoprotein anchored within the apical plasma membrane with the active site of the enzyme facing the colloid [Taurog, 00]. Firstly, the TPO catalyzes the oxidation of iodide. This reaction requires H_2O_2, which is produced by dual oxidases (DUOX1 and 2) that co-localize with TPO [De Deken, 00; Ris-Stalpers, 06]. Secondly, the TPO catalyzes the iodination of tyrosyl residues within the Tg molecule yielding mono- and diiodo-tyrosyl residues (MIT) and (DIT). Finally, the TPO catalyzes the coupling of MIT and DIT, which remain covalently bound to Tg [Taurog, 00]. Coupling of two DIT residues gives rise to T_4 (Figure 1B). Coupling of MIT to the phenolic group of DIT represents the thyroidal biosynthetic pathway of another representative of thyronines which is referred to as L-3,5,3'-triiodothyronine (T_3) (Figure 1C).

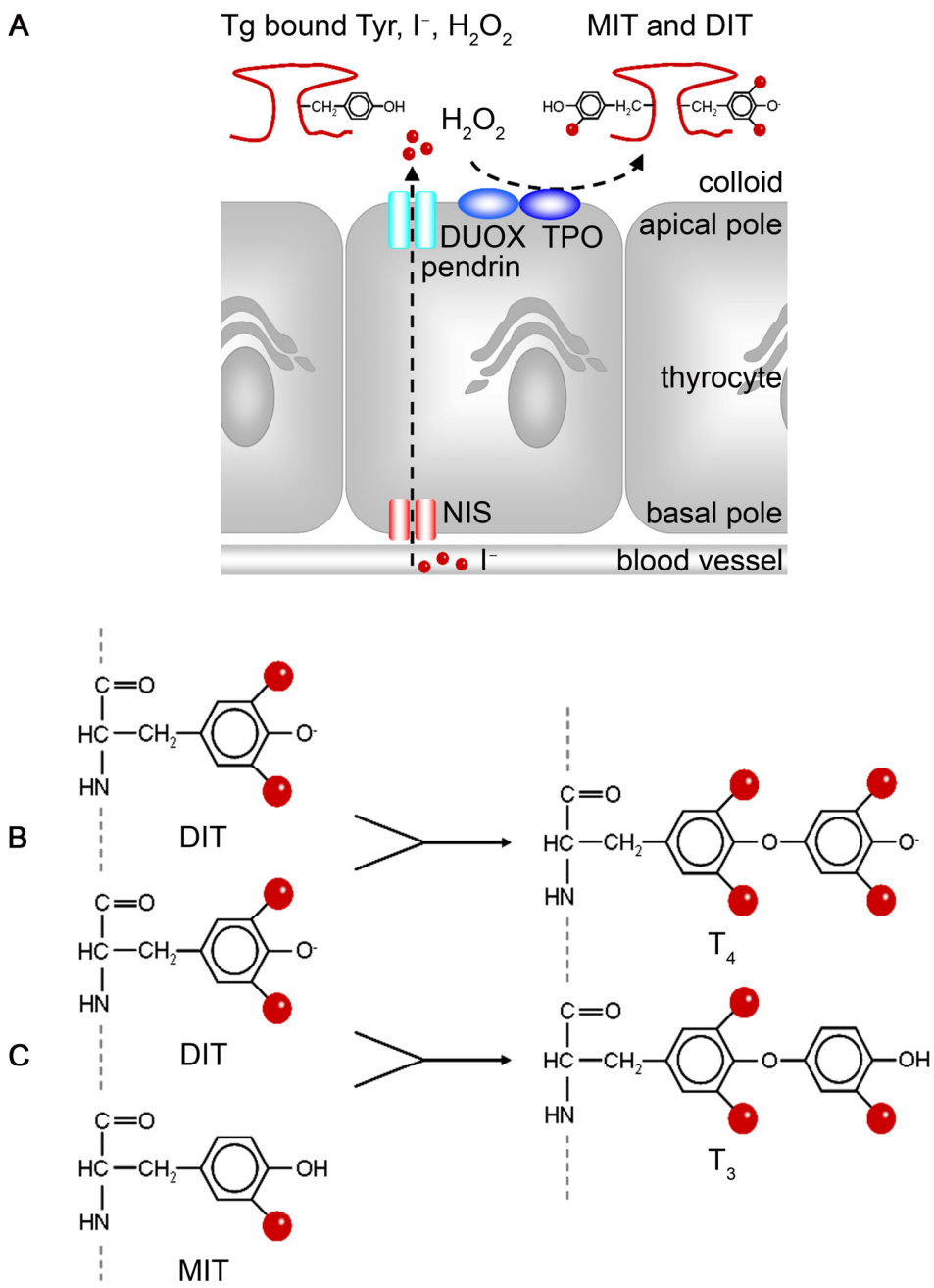


Figure 1: Biosynthesis of T_4 and T_3 in the thyroid gland. Subcellular localization and simplified activities of NIS, pendrin, DUOX and TPO allowing for MIT and DIT biosynthesis within the Tg molecule (**A**). TPO-catalyzed coupling of MIT and DIT residues allowing for biosynthesis of T_4 (**B**) and T_3 (**C**). Partly adopted from [Löffler, 03]. Red spheres: iodide.

The secretion of THs requires the endocytosis of iodinated Tg from the apical thyrocyte plasma membrane [Taurog, 00]. Within the endocytotic vesicles, Tg is proteolytically cleaved, which leads to the release of THs from Tg. THs are secreted into the blood via the basolateral thyrocyte plasma membrane.

The TH biosynthesis within the thyroid gland is exquisitely regulated by the synergistic action of two further hormones, namely TRH (TSH releasing hormone) and TSH (thyroid stimulating hormone, thyrotropin) [Yen, 01]. The fraction of TRH regulating the thyroidal TH biosynthesis is produced in the paraventricular nucleus of the hypothalamus. Composed of the three amino acids pyro-Glu-His-Pro-NH_2, TRH represents the smallest known releasing hormone [Yen, 01]. To activate TSH secretion, TRH is transported from the hypothalamic neurons to the anterior pituitary via the blood of the portal capillary plexus. By binding to its cognate receptor, which is a G_{q11}-protein coupled receptor (G_{q11}PCR) expressed in the plasma membrane of the thyrotropes of the anterior pituitary, TRH activates TSH secretion and *de-novo* synthesis [Yen, 01].

TSH is a heterodimeric glycoprotein composed of an α-subunit, which is identical to the α-subunit of FSH (follicle stimulating hormone), LH (luteinizing hormone) and hCG (human chorionic gonadotropin), as well as a non-covalently linked β-subunit, which is unique to TSH. Upon secretion into the blood, TSH acts as the primary activator of the thyroidal TH biosynthesis by binding to its cognate G_sPCR, which is located to the plasma membrane of thyrocytes [Parmentier, 89]. Activation of the TSH receptor stimulates the expression of genes involved in TH biosynthesis (e.g. Tg, NIS, TPO) and TH secretion. The net activity of this so-called hypothalamus-pituitary-thyroid-axis is regulated by negative feedback loops. TRH biosynthesis and secretion are inhibited by T_3 and TSH [Fekete, 07; Prummel, 04], while TSH biosynthesis and secretion are inhibited by T_3 and TSH itself [Schneider, 01; Prummel, 04].

1.1.2 Biological actions of T_4 and T_3

Secreted THs are transported to their target tissues via the blood stream. In humans, the total T_4 and T_3 blood concentrations are 90 nM and 2 nM, respectively [Schussler, 00]. However, since 99.9 % of T_4 and 99.0 % of T_3 are bound to specific plasma proteins, such as TBG (thyroxine binding globulin), TTR (transthyretin, thyroxin binding prealbumin, TBPA) and albumin (also thyroxine binding albumin, TBA), the free T_4 and T_3 concentrations, which are available to uptake into target cells, are considerably lower [Richardson, 07].

In target cells, THs modulate classical genomic and rapid plasma membrane-initiated signaling pathways to exert their biological effects. To exert genomic actions, THs are transported across the plasma membrane of the target cell by transport proteins, such as MCT8 [Friesema, 06], MCT10 [Visser, 07] or OATP1C1 [Friesema, 05], and further transported into the nucleus where they modulate the activity of nuclear thyroid hormone receptors (TRs) [Oetting, 07]. Since T_3 binds to TRs with much greater affinity than other THs, T_3 is considered as the biologically active thyroid hormone [Hiroi, 06]. TRs belong to the second class of the superfamily of nuclear receptors, which implies that they are mostly constitutively localized to the nucleus and heterodimerize with 9-*cis*-retinoic-X-receptors (RXRs) to bind to the cognate thyroid hormone response elements (TRE) in their target genes promoters [Mangelsdorf, 95]. So far, three TRs have been described, which are encoded by two distinct genes designated as Thra and Thrb [Oetting, 07]. TRα1 is expressed from the Thra gene [Mitsuhashi, 88] while TRβ1 and TRβ2 are expressed from the Thrb gene by alternative splicing [Hodin, 89]. TRα1 and TRβ1 are expressed widely expressed among TH target tissues [Ribeiro, 08], but the expression of TRβ2 is restricted to specific tissues, such as the anterior pituitary, some areas of hypothalamus, developing hippocampus, striatum, chochlea and cone photoreceptors [Hodin, 90; Cook, 92; Bradley, 92; Yanagi, 02; Jones, 07]. Upon activation by T_3 binding, the nuclear TRs modulate the expression of T_3 target genes [Oetting, 07]. Some target genes are positively regulated, such as the cardiac β_1 adrenergic receptor gene [Williams, 77; Ba-

houth, 91], while others are negatively regulated, such as the pituitary TSHβ gene [Bodenner, 91] and the hypothalamic TRH gene [Hollenberg, 95]. So far, no overarching model has become available to explain how T_3 can trigger the activation of one target gene and the repression of a second target gene [Oetting, 07]. Furthermore, in the absence of ligand, TRs do not only bind to TREs but also repress the basal transcription of positively regulated target genes [Chen, 95; Baniahmad, 92].

As mentioned above, THs also exert rapid plasma membrane-initiated effects which are insensitive to transcriptional blockade [Oetting, 07]. Some of these effects are mediated independently from TRs, such as the activation of MAPK upon binding of T_4 or T_3 to plasma membrane bound $\alpha_V\beta_3$ integrin [Bergh, 05]. Interestingly, T_4 was more potent than T_3 at eliciting this effect. Other rapid effects of THs involve the roughly 10 % of TRs which are located outside the nucleus, such as the T_3 dependent interaction between cytosolic TRβ2 and phosphoinositide 3-kinase (PI3K) described for a rat pituitary cell line [Baumann, 01; Storey, 06].

The biological effects attributed to thyroid hormones appear very complex and cannot be addressed completely herein. Throughout the vertebrate kingdom, thyroid hormones are critically involved in the regulation of ontogenic development and growth of various organs, such as brain [Silva, 03]. In homeothermic species, they additionally acquired a role in metabolic regulation including thermogenesis [Kim, 08]. Thyroid hormones increase adenosine triphosphate (ATP) consumption by activating the expression of Na^+/K^+ ATPases and sarcoplasmatic Ca^{2+} ATPases as well as by accelerating practically all anabolic and catabolic pathways [Ribeiro, 08; Silva, 03]. The latter effect does not only account for their metabolic actions whose net effects are lipogenesis and increased food intake but also for their obligatory hyperthermic function [Silva, 03]. Thyroid hormones increase the proton leak of the inner mitochondrial membrane thus reducing the efficiency of ATP synthesis and allowing energy dissipation as heat [Harper, 93]. Locally produced T_3 is also essential to mediate facultative thermogenesis in brown adipose tissue upon cold exposure [Bianco, 87]. Finally, T_3 has positive inotropic and

chronotropic effects on the heart and lowers peripheral vascular resistance, which altogether leads to an increased cardiac output [Klein, 01].

1.2 Deiodinases

As outlined above (section 1.1.1), T_4 is exclusively biosynthesized *de-novo* in the thyroid gland [Engler, 84]. By contrast, T_3 is either produced *de-novo* within the Tg molecule or by enzymatic deiodination of T_4 at the phenolic ring [Köhrle, 02].

Deiodination reactions imply the sequential reductive removal of iodine from the precursor molecule. They are catalyzed by a group of three isozymes designated as iodothyronine deiodinases (Dio1, 2 and 3). Dio1 exhibits both phenolic and tyrosyl ring deiodination activity [Köhrle, 02] Figure 2). Phenolic ring deiodinations are also referred to as 5'- and 3'-deiodinations, depending on whether the substrate possesses two or one iodine atoms in the phenolic ring, respectively. Accordingly, tyrosyl ring deiodinations are also referred to as 5- and 3-deiodinations (Figure 2). Both the 5'- and 3'-position as well as the 5- and 3-position are chemically equal [Köhrle, 84]. In contrast to Dio1, Dio2 and Dio3 are more specific with respect to the position of the iodine removed. Dio2 catalyzes only deiodinations of the phenolic ring, e.g. the conversion of T_4 to T_3 [Köhrle, 02] Figure 2). Due to this biosynthetic pathway of T_3 as a T_4 metabolite and due to the finding that T_3 binds to thyroid hormone receptors with greater affinity than T_4 [Hiroi, 06], T_3 is considered as the biologically active thyroid hormone whereas T_4 is considered as a prohormone [Moreno, 08]. Dio3 catalyzes only deiodinations of the tyrosyl ring, e.g. the conversion of T_4 to another representative of thyronines which is referred to as L-3,3',5'-T_3 (reverse T_3, rT_3) [Köhrle, 02] Figure 2). Since no definite biological function has been elucidated for rT_3, so far, it is considered as an inactive T_4 metabolite rather than a thyroid hormone [Moreno, 08].

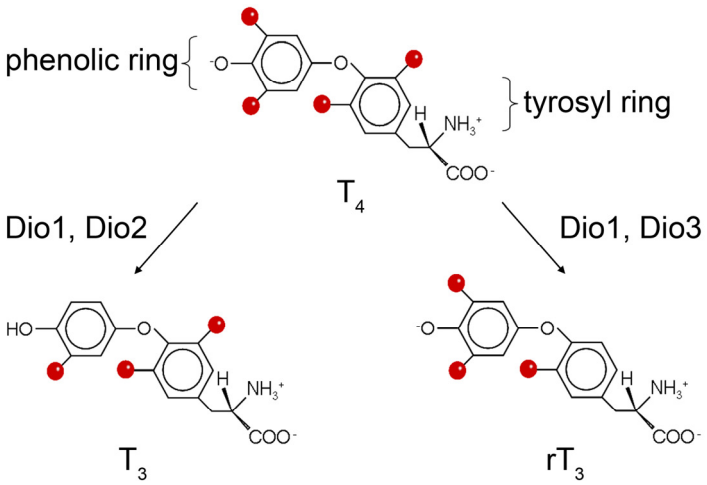
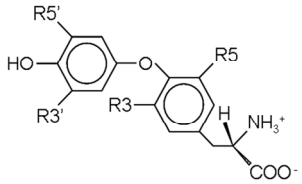


Figure 2: Catalytic properties of Dio isozymes. Dio1 catalyzes both phenolic and tyrosyl ring deiodinations whereas Dio2 and Dio3 catalyze only one type of deiodination as indicated.

However, not only T_4 but also T_3 and rT_3 are substrates of deiodination reactions yielding further representatives of the group of thyronines, namely L-diiodothyronines (3,5-T_2, 3,3'-T_2, 3',5'-T_2), L-monoiodothyronines (3-T_1, 3'-T_1) and L-thyronine (T_0) [Chopra, 78b] Figure 3). Though T_2s, T_1s and T_0 have been detected *in-vivo* [Chopra, 78a; Wu, 76; Meinhold, 78; Maciel, 79; Smallridge, 79; Corcoran, 83; Willetts, 79; Ramsden, 84], their biological functions remain elusive. Thus, so far, deiodinations of T_3 have been interpreted as a reactions degrading biologically active thyroid hormone rather than as reactions biosynthesizing another TH. Accordingly, controlling the local and systemic bioavailability and thus the action of T_3 has been considered as the main function of Dio isozymes [Köhrle, 02].



TH	R3	R5	R3'	R5'
T_4	I	I	I	I
rT_3	I	H	I	I
T_3	I	I	I	H
3',5'-T_2	H	H	I	I
3,3'-T_2	I	H	I	H
3,5-T_2	I	I	H	H
3'-T_1	H	I	H	H
3-T_1	I	H	H	H
T_0	H	H	H	H

Figure 3: Structure and nomenclature of the group of thyronines (THs). R: variable residue, I: iodine, H: hydrogen.

In all species cloned to date, the Dio isozymes contain the amino acid selenocysteine (Sec) as part of a highly similar active center and a Sec insertion sequence (SECIS) in their genes [Köhrle, 02]. Thus, Dio isozymes are representatives of selenoproteins, which require a more complex translation machinery compared to typical non-selenoproteins [Köhrle, 05].

Despite this structural similarity and their concerted function in regulating thyroid hormone bioavailability, the Dio isozymes differ considerably in terms of catalytic properties, substrate specificities, kinetic properties, mechanisms of reaction, expression patterns, physiological functions and pathophysiological implications [Bianco, 02].

Since this study was focused on the identification of new Dio substrates and the determination of their kinetic parameters, the published substrate specificities of the Dio isozymes towards THs are summarized in Figure 4.

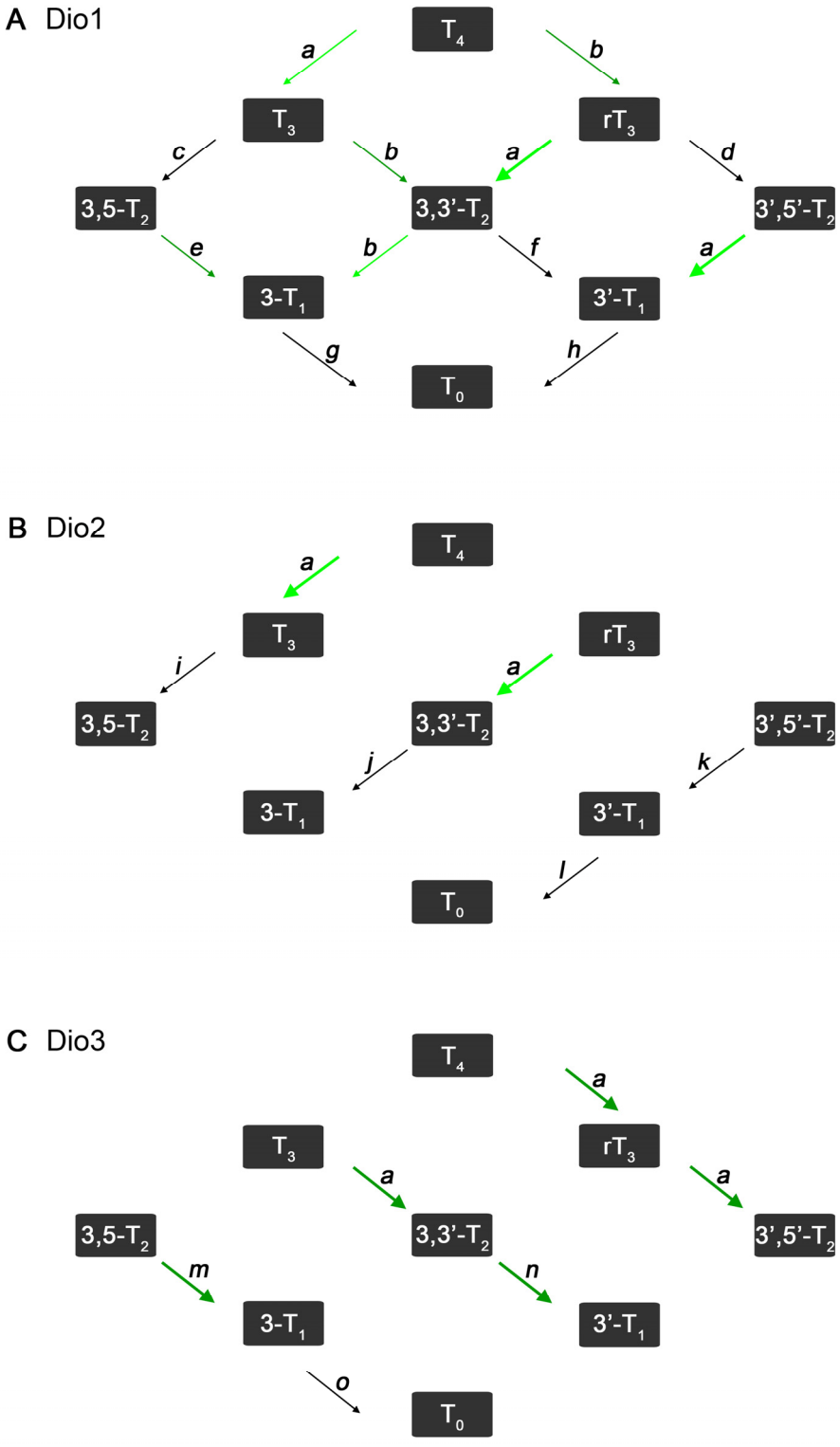

A Dio1
T4
a
b
T3
rT3
c
b
a
d
3,5-T2
3,3'-T2
3',5'-T2
e
b
f
a
3-T1
3'-T1
g
h
T0
B Dio2
T4
a
T3
rT3
i
a
3,5-T2
3,3'-T2
3',5'-T2
j
k
3-T1
3'-T1
l
T0
C Dio3
T4
a
T3
rT3
a
a
3,5-T2
3,3'-T2
3',5'-T2
m
n
3-T1
3'-T1
o
T0

Figure 4: Substrate specificities of Dio1 (**A**), Dio2 (**B**) and Dio3 (**C**) towards THs. Arrows: pointing to lower left: phenolic ring deiodinations, pointing to lower right: tyrosyl ring deiodinations, light green: phenolic ring deiodinations catalyzed by the respective Dio isozyme, dark green: tyrosyl ring deiodinations catalyzed by the respective Dio isozyme, black: deiodination reactions which were either yet or found not to be catalyzed by the respective Dio isozyme.

[a] [Köhrle, 02]

[b] [Kuiper, 05; Wu, 05]

[c] Several studies have excluded T_3 as a substrate of phenolic ring deiodination catalyzed by Dio1 [Sorimachi, 77; Höffken, 78a; Visser, 78; Kaplan, 78; Moreno, 08]. However, two studies have reported that homogenates of the NCLP-6E monkey hepatocarcinoma cell line, which expresses Dio1 enzymatic activity [Sorimachi, 79b], catalyze the phenolic ring deiodination of T_3 [Sorimachi, 79a; Sorimachi, 79b]. Nevertheless, these findings were referred to as unpublished data and have never been officially published, so far.

[d] 3',5'-T_2 was detected in serum of euthyroid human subjects upon a bolus injection of [5'-^{125}I]rT_3 [Rudolph, 78; Sakurada, 78; Lumholtz, 78]. However, since euthyroid humans express both Dio1 and Dio3 enzymatic activity, the observed tyrosyl ring deiodination of rT_3 cannot be assigned unambiguously to either Dio isozyme as no isozyme specific inhibitors, such as 6-n-propyl-2-thiouracil (PTU) (see section 1.2.1), were included in the study.

[e] [Chopra, 82]

[f] excluded [Sorimachi, 80a; Otten, 84]

[g] excluded [Chopra, 81]

[h] In some studies 3'-T_1 was not deiodinated to T_0 by Dio1 preparations [Sorimachi, 80a; Otten, 84; Chopra, 81] while in other studies weak 3'-T_1 degradations were observed by Dio1 preparations [Smallridge, 81; Smallridge, 82; Smallridge, 84]. However, T_0 formation was measured in any of these studies.

[i] not reported, so far

[j] In the only study reporting on the phenolic ring deiodination of 3,3'-T_2 by Dio2 containing preparations [Chopra, 81], 3,3'-T_2 was not 3'-deiodinated by rat brain homogenates. However, this study appears inappropriate to exclude 3,3'-T_2 as a Dio2 substrate since rat brain expresses both Dio1 and Dio2 enzymatic activity [Bates, 99] and should thus have 3'-deiodinated 3,3'-T_2 anyway.

[k] Although serum 3'-T_1 concentrations increased in human subjects receiving an intravenous infusion of 3',5'-T_2, the observed tyrosyl ring deiodination of 3',5'-T_2 cannot be assigned unambiguously to Dio2 since no isozyme specific inhibitors, such as PTU (see section 1.2.1), were included in the study to discriminate between the two phenolic ring deiodination activities expressed in humans, namely Dio1 and Dio2. In the same study cited under [j] [Chopra, 81], rat brain homogenates, which contain both Dio1 and Dio2 [Bates, 99], did not 5'-deiodinate 3',5'-T_2. As delineated above, this study was not appropriate to unequivocally exclude 3',5'-T_2 as a substrate of Dio2.

[l] excluded [Smallridge, 84]

[m] [Sorimachi, 79a; Sorimachi, 79b]

[n] [Sorimachi, 77; Kaplan, 80]

[o] So far, the tyrosyl ring deiodination of 3-T_1 to T_0 by Dio3 has not been demonstrated unequivocally, i.e. by experiments demonstrating both the disappearance of 3-T_1 and the formation of T_0. One study, which demonstrated the monodeiodination and sulfation of [3,5-^{125}I]T_2 by Dio3 expressing intact cultured monkey hepatocarcinoma NCLP-6E cells, provided indirect evidence for a tyrosyl ring deiodination of 3,3'-T_2 by Dio3 since the production rate of $^{125}I^-$ was slightly greater than that of [3-^{125}I]T_1 [Sorimachi, 79a]. However, since small amounts of unidentified TH sulfates were detected, it cannot be excluded that the free $^{125}I^-$ was released from sulfated [3-^{125}I]T_1.

1.2.1 Dio1

Dio1 is the only isozyme that exhibits both phenolic and tyrosyl ring deiodination activity. With iodothyronine substrates, K_m values in the higher nanomolar to lower micromolar range and high v_{max} values (pmol/mg/min) were measured. In terms of v_{max}/K_m ratios, rT_3 is the preferred substrate of Dio1 [Köhrle, 02]. The deiodination reactions catalyzed by Dio1 follow ping pong kinetics with two substrates [Kuiper, 05]. In the first half reaction, the iodothyronine substrate transfers an I^+ to the selenolate anion (E-Se^-) of the Sec within the active center thus forming a putative selenenyl iodide intermediate (E-Se-I). In the second half reaction, this intermediate is reduced by an intracellular cosubstrate, which concomitantly regenerates the Sec residue of the enzyme. The monodeiodinated product and I^- are released. *In-vitro*, the E-Se-I intermediate is efficiently reduced by dithiols, such as dithiothreitol (DTT) and dithioerythritol (DTE) [Köhrle, 02]. However, the putative physiologic cosubstrate regenerating Dio1 has not been identified yet. Moreover, the hypothesis that a productive deiodination reaction catalyzed by Dio1 might lead to suicide inactivation of Dio1 without regeneration of the active enzyme *in-vivo*, has not been rejected, so far [Köhrle, 02]. Another hallmark characteristic of Dio1, which allowed for its early distinction from Dio2, was the inhibition of its catalytic activity by 6-n-propyl-2-thiouracil (PTU) [Oppenheimer, 72; Hesch, 75]. PTU inhibition of Dio1 is uncompetitive with the iodothyronine sub-

strate, and there is strong evidence that PTU binds to the E-Se-I intermediate thus forming a selenenyl sulfide (E-Se-S-PTU), which is considered as a dead-end complex [Visser, 88; du Mont, 01].

In humans, Dio1 mRNA and enzymatic activity were detected in the thyroid gland and in classical T_3 target tissues, such as pituitary, liver, kidney and heart [Köhrle, 02; Bianco, 02]. Dio1 mRNA was also detected in circulating mononuclear cells [Nishikawa, 98]. In contrast to rats, Dio1 enzymatic activity was notably absent from the human central nervous system [Campos-Barros, 96]. So far, no coherent data have been reported on the subcellular localization of the mature Dio1 protein. In liver, Dio1 was located to the cytosolic surface of the endoplasmatic reticulum [Auf Dem Brinke, 79; Fekkes, 79; Fekkes, 80]. By contrast, in epithelial kidney cells, Dio1 was detected at the basolateral plasma membrane [Leonard, 78; Leonard, 91; Leonard, 01].

Supplying a significant fraction of plasma T_3 synthesized from T_4 has long been considered as the pivotal biological function of Dio1 [Bianco, 02]. Plasma T_3 is thought to be secreted from the thyroid gland but also to originate from peripheral 5'-deiodination of T_4, which could be catalyzed either by Dio1 or Dio2. However, a role of Dio1 was challenged by the unchanged serum T_3 levels of mice carrying a targeted disruption of the Dio1 gene or mice genetically deficient in hepatic selenoprotein translation due to albumin-driven knock-out of the $tRNA^{Ser(Sec)}$ (Trsp) gene [Schneider, 06; Streckfuss, 05]. So far, the biological function of Dio1 has not been demonstrated unequivocally. Considering that the v_{max}/K_m ratios for the Dio1 catalyzed 5'-deiodination of rT_3 and 3',5'-T_2 are much higher than that for T_4, its catalytic action with respect to these substrates might reflect its major physiological function [Köhrle, 84; Bianco, 02].

1.2.2 Dio2

Dio2 catalyzes only deiodinations of the phenolic ring. Its catalytic activity is clearly distinguishable from the phenolic ring deiodination activity of Dio1 since

Dio2 exhibits K_m values in the lower nanomolar range and lower v_{max} values than Dio1. In terms of v_{max}/K_m ratios, T_4 represents the preferred substrate of Dio2 [Köhrle, 02]. Its catalytic activity is insensitive to PTU. Deiodination reactions catalyzed by Dio2 follow sequential kinetics suggesting that both the iodothyronine substrate and the cosubstrate must interact with the enzyme before the reaction takes place [Visser, 82; Köhrle, 02]. The deiodination of an iodothyronine by Dio2 requires an endogenous reducing cosubstrate. Its identity remains elusive, but DTT works efficiently *in-vitro* [Köhrle, 02].

The Dio2 expression pattern is species-specific indicating that the enzyme might have species-specific biological functions [Bianco, 02]. Dio2 mRNA and/or enzymatic activity are expressed in the human thyroid gland, heart, kidney, pancreas, skeletal muscle, brain, spinal cord, keratinocytes and placenta [Salvatore, 96b; Murakami, 01; Salvatore, 96a; Bartha, 00; Croteau, 96; Campos-Barros, 96; Kaplan, 88]. By contrast, in rats, Dio2 enzymatic activity was not detected in the adult thyroid gland and heart [Bates, 99; Croteau, 96] but present in the neonatal thyroid gland as well as pituitary, brain, brown adipose tissue, pineal gland, thymus, gonads and pregnant uterus [Bates, 99; Silva, 77; Cheron, 79; Visser, 82; Leonard, 88; Silva, 83; Kamiya, 99; Molinero, 95; Galton, 01]. Thus, Dio2 represents the only phenolic ring Dio activity in the adult human brain, whereas both Dio1 and Dio2 activity were detected in rat brain. In cell lines, both endogenous and transiently expressed FLAG-tagged Dio2 localized to the endoplasmatic reticulum with the catalytic center facing the cytosol [Baqui, 00; Curcio, 01].

Since the 5'-deiodination of T_4 to T_3 is the preferred reaction catalyzed by Dio2, this isozyme is assumed to generate biologically active T_3 from local T_4 sources for intracellular demands independently from circulating T_3 [Köhrle, 05]. In serum of euthyroid mice carrying a targeted disruption of the Dio2 gene, T_4 and TSH concentrations were elevated, but T_3 concentrations were normal suggesting that the pituitary thyrotropes were not responding normally to the serum T_4 concentration. These findings suggested that Dio2 is critically required for the feedback inhibition of TSH synthesis by plasma T_4 [Schneider, 01].

1.2.3 Dio3

Dio3 catalyzes deiodinations of the tyrosyl ring, exclusively [Köhrle, 02]. Depending on the experimental enzyme source used, widely ranging apparent K_m and v_{max} values were reported for the tyrosyl ring deiodination of T_4 and T_3 by Dio3. Maximum enzymatic activity required high concentrations of DTT, but the endogenous cofactor(s) has (have) not been identified [Kaplan, 80]. Dio3 enzymatic activity was observed to be insensitive towards PTU [Croteau, 95].

In adulthood, Dio3 activity is expressed in brain of humans and rats as well as in skin, eye and gonads of rats [Campos-Barros, 96; Kaplan, 80; McCann, 84; Huang, 85; Bates, 99; Huang, 05]. However, much higher Dio3 expression has been demonstrated in fetal tissues, such as brain, liver and intestine [Chan, 02; Kester, 04; Richard, 98; Galton, 91; Bates, 99] as well as at sites of the maternal-fetal interface, such as uterus, placenta and umbilical arteries and veins [Huang, 03; Galton, 99; Koopdonk-Kool, 96; Roti, 82; Bates, 99; Hernandez, 06]. Developmental profiles of Dio3 expression have been investigated for many tissues in various species [Köhrle, 02; references 162 - 171]. Dio3 activity was located to the microsomal subcellular fraction of several tissues [Höffken, 78b; Schoenmakers, 95], and demonstrated as a phospholipid requiring enzyme [Santini, 92]. However, the definite subcellular location and topology have not been reported, so far [Bianco, 02].

Dio3 contributes to thyroid hormone homeostasis but in contrast to Dio2 as a thyroid hormone inactivating enzyme, which is supported by the hypothyroidism in patients with Dio3 overexpressing hepatic hemangiomas [Huang, 00]. A function of Dio3 in protecting tissues from an excess of T_4 and T_3 actions has been widely accepted [Hernandez, 06]. This was proven true for embryogenesis, where excess or premature exposure of the embryo to maternal thyroid hormone levels can be detrimental, e.g. by the growth retardation, neonatal mortality and abnormal thyroid status of the Dio3 knock-out mouse [Hernandez, 06] or by the blockade of metamorphosis in Xenopus *laevis* tadpoles overexpressing Dio3 [Huang,

99]. However, such a function of Dio3 remains speculative for adult organisms [Hernandez, 06].

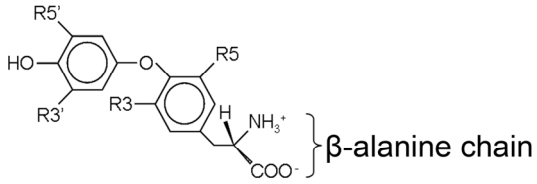
1.3 Thyronamines

The history of thyronamine (TAM) research seems peculiar. From 1951 to 1984, several papers were published every now and then, which were mostly lacking any topical consistency. In 2004, after a twenty year gap, TAMs aroused renewed interest due to a publication by Thomas S. Scanlan and co-workers [Scanlan, 04]. Ever since, the field of TAM research has become very vivid again.

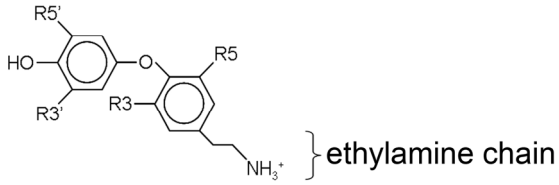
1.3.1 Structure of TAMs

TAMs are a novel class of endogenous signaling compounds. In terms of structure, they differ from THs only concerning the absence of the carboxylate group of the β-alanine side chain. Following the same rules for nomenclature as THs, TAMs are designated T_xAM, with x indicating the number of iodine atoms per molecule (Figure 5). Accordingly, there are nine TAMs differing either by the number or by the positions of the iodine atoms.

A thyronine (TH)



B thyronamine (TAM)



TAM	R3	R5	R3'	R5'
T_4AM	I	I	I	I
rT_3AM	I	H	I	I
T_3AM	I	I	I	H
3',5'-T_2AM	H	H	I	I
3,3'-T_2AM	I	H	I	H
3,5-T_2AM	I	I	H	H
3'-T_1AM	H	I	H	H
3-T_1AM	I	H	H	H
T_0AM	H	H	H	H

Figure 5: Structure of thyronines (THs) (**A**). Structure and nomenclature of thyronamines (TAMs) (**B**). R: variable residue, I: iodine, H: hydrogen.

1.3.2 *In-vivo* detection of TAMs

So far, only two representatives of TAMs, namely 3-T_1AM and T_0AM, have been detected *in-vivo* using an LC-MS/MS technique [Scanlan, 04]. Both compounds were measured in blood, heart, liver and brain of C57BL/6 adult male mice as well as in brain of several other species, such as rats and guinea pigs [Scanlan, 04]. 3-T_1AM was also detected in blood and brain of Djungarian hamsters [Braulke, 08].

The presence of other TAMs *in-vivo* has not been proven yet. In the LC-MS/MS studies by [Scanlan, 04] the samples were simultaneously monitored for T_0AM,

T_1AM and T_2AM, but neither T_2AM isomer was detected in any of the tissues. Likewise, T_3AM has not been detected, so far [Meyer, 83]. In early experiments applying butanolic extraction and subsequent paper chromatographic separation, ^{131}I labeled T_4AM was found in plasma and in the thyroid glands but not in the thyroid derived Tg fractions of rats which had been treated with ^{131}I [HILLMANN, 58]. Furthermore, ^{131}I labeled T_4AM but no T_3AM was detected in the plasma of two patients who suffered from metastasizing thyroid tumors and were treated with high dosages of ^{131}I. Based on these experiments, T_4AM was postulated to be present *in-vivo* at a serum concentration equalling 1 to 2 % of the serum T_4 concentration [HILLMANN, 58; HILLMANN, 61]. However, T_4AM biosynthesized from endogenous precursors has not yet been detected yet.

Hitherto, few publications have reported on endogenous TAM concentrations in tissues. 3-T_1AM was estimated to be present in rat brain at subpicomole per gram quantities [Scanlan, 04]. This concentration is lower compared to 1 to 6 pmol/g T_4 which have been reported for rat brain using a radioimmunoassay technique [Pinna, 02]. For rat heart, an endogenous 3-T_1AM concentration of 68 pmol/g wet weight was calculated [Chiellini, 07]. Interestingly, this concentration corresponds to the cardiac content of classical modulators of heart function, such as adrenaline, dopamine and adenosine [Chiellini, 07]. Moreover, it exceeds the endogenous intracardial T_3 and T_4 concentrations by factor 20 and 2, respectively [Chiellini, 07]. Even if it was not stated whether the hearts had been perfused, these findings indicate that TAMs can override TH concentrations locally in the heart. In the serum of Djungarian hamsters either adapted to long photoperiods (LP) or short photoperiods (SP), endogenous 3-T_1AM was detected at a concentration of roughly 5 nM [Braulke, 08].

1.3.3 Physiological effects of TAMs

The physiological roles of TAMs remain elusive. In early studies, T_4AM and T_3AM were only 10 % as potent as T_4 in terms of goiter prevention and stimulation of

oxygen consumption in rats [TOMITA, 56; THIBAULT, 51]. However, T_3AM was 36 times as potent as T_4 at inducing tadpole metamorphosis [TOMITA, 56]. In more recent studies, intraperitoneal injection of 3-T_1AM or T_0AM into C57BL/6 wild type mice or Djungarian hamsters caused various prompt effects, such as hypothermia, metabolic repression, hyperglycemia, reduction of the respiratory quotient, negative chronotropy and negative inotropy [Scanlan, 04; Braulke, 08]. In most of these studies, a dose of 50 mg 3-T_1AM or T_0AM/kg body weight was injected into animals. In Djungarian hamsters, this dosage lead to an approximately tenfold increase in 3-T_1AM blood concentration [Braulke, 08]. To this end, it is not known whether such changes in TAM blood and tissue concentrations have to be considered as physiological or pharmacological. Therefore, the effects of TAMs described so far, will conditionally be classified and described as pharmacological effects, herein.

1.3.4 Pharmacological effects of TAMs

Upon intraperitoneal injection into C57BL/6 wild-type mice, 3-T_1AM and T_0AM reduced the body temperature within 30 min by roughly 8 °C at a half maximal effective concentration (EC_{50}) of 59 and 178 µmol/kg body weight, respectively [Scanlan, 04]. The mice became inactive, but reflexes were retained indicating that they were not anesthetized. Furthermore, their skin cooled off, but they did not display any compensatory reactions, such as shivering, huddling or piloerection. The effect was completely reversible after 6 to 8 hours and so far, no side effects were reported even after repeated treatments over a period of 2 months [Scanlan, 04]. Although less pronounced, this hypothermic effect was also observed after intraperitoneal injection of 3-T_1AM into Djungarian hamsters [Braulke, 08].

In wild type C57BL/6 mice and in Djungarian hamsters, the hypothermia caused by 3-T_1AM injection was temporarily preceded by a reduction in metabolic rate [Braulke, 08]. Since the extent of hypothermia and the amount of metabolic re-

duction were highly correlated, the hypothermic effect was interpreted as a result of the reduced metabolic rate [Braulke, 08].

In addition to hypothermia and metabolic repression, wild type mice intraperitoneally injected with 3-T_1AM displayed an increase in blood glucose level [Regard, 07]. This hyperglycemic effect was detectable within minutes after treatment, reaching a maximum of 250 % above basal 2 h after 3-T_1AM injection and was completely reversed 8 h post-injection. It was accompanied by a decrease in plasma insulin levels and increase in plasma glucagon levels. Insulin applied exogenously 2 h after 3-T_1AM injection normalized the blood glucose levels indicating that the peripheral tissues had remained sensitive to insulin during the 3-T_1AM treatment [Regard, 07].

Also within minutes after intraperitoneal 3-T_1AM injection, the respiratory quotient (RQ) decreased from 0.9 to 0.7 both in wild type C57BL/6 mice and in Djungarian hamsters [Braulke, 08]. This was indicative of a rapid change in metabolic fuel utilization from predominantly carbohydrates (RQ ~ 0.9) before treatment to primarily lipids after treatment (RQ ~ 0.7) [Braulke, 08]. Minimal RQ values were obtained 3 to 4.5 h post-treatment. The reduced RQ was maintained for more than 24 h, i.e. even after the recovery of metabolic rate and return to normothermia. This indicated that the treated animals required several hours to readjust their metabolic machinery to normal catabolism [Braulke, 08].

The hypothermic and metabolic effects of 3-T_1AM intraperitoneally injected into wild type C57BL/6 mice were accompanied by rapid and reversible bradycardia [Scanlan, 04]. This effect was direct since it was reproducible by perfusing an *ex-vivo* adult rat denervated working heart preparation with 3-T_1AM. At concentrations too low to elicit bradycardia, 3-T_1AM decreased the cardiac output within minutes at an EC_{50} of 29 µM [Scanlan, 04].

Conflicting data have been reported for the cardiac effects of T_0AM. In the perfused, denerved rat heart, T_0AM decreased the cardiac output within minutes at an EC_{50} of 83 µM while an effect on the heart rate was not reported [Scanlan, 04].

By contrast, using an isolated guinea pig atria model, T_0AM (2.9 nM to 9.5 µM) displayed positive inotropic effects and did not modify the heart rate [Boissier, 73]. In anesthetized rats, T_0AM (3 to 30 mg/kg body weight) decreased the heart rate significantly [Boissier, 73]. Upon intravenous injection into anesthetized dogs, T_0AM increased the cardiac output [Boissier, 73; Cote, 74]. However, T_0AM induced concomitant negative chronotropy in one study [Boissier, 73] but positive chronotropy in the other study [Cote, 74].

1.3.5 Receptors for TAMs

To this end, the physiological receptor(s) of TAMs has (have) not been identified unambiguously. While some receptors have been excluded as candidates, scattered experimental evidence has been gathered for other receptors to mediate TAM signaling.

The structural similarities of TAMs with THs and biogenic amines suggested that TAMs might signal via nuclear thyroid hormone receptors or plasma membrane-bound receptors of biogenic amines. But considering the rapid onset of TAMs effects, the latter pathway appeared more likely. However, T_0AM and 3-T_1AM were reported neither to bind to nor to activate nuclear thyroid hormone receptors using nuclear receptor binding experiments and reporter gene assays, respectively [Scanlan, 04]. Furthermore, neither T_0AM nor 3-T_1AM activated the heterologously expressed $G_{\alpha s}$-protein coupled dopamine D_1 receptor and β_2 adrenergic receptor [Scanlan, 04]. Thus, despite structural similarities, TAMs, THs and certain biogenic amines appear to signal via different receptors.

The first receptor suggested to mediate TAM effects, was the membrane bound G-protein coupled trace amine associated receptor 1 (TAAR-1) [Scanlan, 04]. This suggestion was based on the finding that 3-T_1AM, 3,3'-T_2AM, 3,5-T_2AM, T_3AM and T_0AM coupled the heterologously expressed rat TAAR-1 to cyclic adenosine monophosphate (cAMP) biosynthesis at EC_{50} values ranging from 14 to 131 nM with respect to the indicated order [Scanlan, 04]. Given the structural

similarity between TAMs and biogenic amines as well as the expression of TAAR-1 transcript in TAM target tissues, such as heart [Chiellini, 07] and pancreas [Regard, 07], TAAR-1 has been considered as an excellent candidate receptor for T_0AM and 3-T_1AM [Doyle, 07]. However, the concept of TAM signalling via a $G_{\alpha s}PCR$, such as TAAR-1, was challenged by experiments demonstrating that the negative chronotropic, negative inotropic and hyperglycemic effects of 3-T_1AM were more consistent with an inhibition of cAMP accumulation through $G_{\alpha i}$ activation [Chiellini, 07; Regard, 07]. Yet, TAAR-1 has not been withdrawn as a candidate TAM receptor [Doyle, 07].

A second receptor proposed to mediate the effects of 3-T_1AMs, was the α_{2A} adrenergic receptor (Adra2a), which is an established $G_{\alpha i}PCR$ inter alia expressed in pancreatic β-cells [Regard, 07; Bylund, 88]. *In-vitro* studies demonstrated a higher affinity of 3-T_1AM for Adra2a compared to epinephrine [Regard, 07]. Coadministration of the Adra2a antagonist yohimbine with 3-T_1AM inhibited the hyperglycemic effects of 3-T_1AM. Moreover, 3-T_1AM failed to induce hyperglycemia in Adra2a-null mice [Regard, 07].

1.3.6 Signal transduction pathways activated by TAMs

To assess the signaling pathways activated by TAMs in the heart, *ex-vivo* adult rat working heart preparations, which had been pretreated with various inhibitors of signaling cascades, were perfused with T_0AM and 3-T_1AM [Chiellini, 07]. Remarkably, *Bordetella pertussis* toxin (PTX), an inhibitor of $G_{\alpha i}$ signaling did not interfere with the negative chronotropic effects of 3-T_1AM thus excluding a role of $G_{\alpha i}$ proteins in mediating the effects of 3-T_1AM on the heart. Instead, tyrosine phosphorylations and dephosphorylations were detected in the cytosolic and microsomal fraction of rat ventricles perfused with 3-T_1AM suggesting that 3-T_1AM might signal via tyrosine phosphorylation and dephosphorylation pathways [Chiellini, 07].

1.3.7 Therapeutic applications of TAMs

In adult C57BL/6 mice injected intraperitoneally with 3-T_1AM or T_0AM either 1 or 2 h after or 2 days prior to the experimental induction of stroke, the infarct volumes were reduced compared to vehicle treated control mice [Doyle, 07]. The effect was abolished when the TAM treated mice were placed on a heat pad. This indicated that the neuroprotective effect of TAMs required the induction of hypothermia [Doyle, 07].

1.3.8 Metabolism of TAMs

So far, only one study has investigated potential metabolizing reactions of TAMs [Pietsch, 07]. T_0AM and 3-T_1AM, which are present *in-vivo*, but also T_3AM were found to be readily sulfated by distinct human liver sulfotransferase (SULT) isoforms. Moreover, 3-T_1AM was sulfated by homogenates of human brain and cardiac tissue, i.e. target tissues of TAM action. These SULT actions might serve to attenuate and thus regulate TAM action [Pietsch, 07].

1.3.9 Biosynthesis of TAMs

The pharmacological effects reported for TAMs identified an interesting discrepancy. On the one hand, TAMs and THs are highly similar in structure (Figure 5), but on the other hand, the short-term hypothermic, negative chronotropic and negative inotropic effects of TAMs are opposite in direction to the actions of the classical thyroid hormone T_3. Thus, TAMs were suggested to be derivatives of thyroid hormones that might serve to fine-tune or even antagonize thyroid hormone effects [Liggett, 04].

If TAMs were derivatives of THs, decarboxylation of the β-alanine side chain would be required for their biosynthesis. However, so far, no iodothyronine decarboxylating enzyme has been identified [Wu, 05]. If the putative decarboxylating enzyme was converting only THs with higher iodine content, such as T_4, T_3s or

T_2s, Dio isozymes would be directly required to complete the biosynthesis of 3-T_1AM and T_0AM by removing one to four iodine atoms. However, any role of Dio isozymes in TAM biosynthesis presupposes their ability to accept TAMs as substrates.

1.4 Aims of the study

In the present study, the ability of the Dio isozymes to accept TAMs as substrates was investigated.

The various TAMs analyzed here differed only regarding the number or the position of the iodine atoms. Therefore, an important first objective of this study was to establish a liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to allow for the unequivocal, simultaneous identification and quantification of all TAMs in the same sample. So far, no reference method for TAM quantification has been available. Thus, a further objective of this study was to include the complete panel of THs as established Dio substrates into the LC-MS/MS protocol. This would allow for validating the performance of the LC-MS/MS method in quantifying Dio catalyzed reactions by comparing the kinetic constants of TH deiodination reactions obtained by LC-MS/MS to that obtained by independent methods, such as $^{125}I^-$ release assays or HPLC based radiochemical assays.

The LC-MS/MS based analysis of TAMs and THs from Dio reaction mixtures required their prior extraction by a method compatible with LC-MS/MS measurements. Thus, another aim of this study was to develop an appropriate extraction method allowing for the simultaneous isolation of all TAMs and THs from Dio reaction mixtures. Further experiments aimed at validating the detection of extracted TAMs and THs by LC-MS/MS according to internationally accepted recommendations.

The central objective of this study was to analyze the substrate specificities of the Dio isozymes towards iodothyronamines by independent methods, namely LC-MS/MS based Dio assays and $^{125}I^-$ release assays. Since these analyses required isozyme specific Dio preparations, preliminary experiments were performed to characterize the Dio expression pattern in cell line and tissue sources. Further LC-MS/MS based Dio assays aimed at characterizing the kinetic properties of selected TAM deiodination reactions and comparing those to the corresponding THs.

2 Materials and Methods

2.1 Chemicals and reagents

The chemicals and reagents used in this study were obtained at the highest purity available and are listed in Table 1.

Table 1: List of chemicals and reagents used in this study.

Chemical	Supplier
[5'-^{125}I]rT_3, 250 µCi	Perkin-Elmer, Jügesheim, Germany
3,3'-T_2	formula., Berlin, Germany
3,3'-T_2AM	OHSU, Portland, Oregon, USA [a]
3,5,3'-T_3	formula., Berlin, Germany
3,5,3'-T_3AM	formula., Berlin, Germany
3,5-T_2	formula., Berlin, Germany
3,5-T_2AM	OHSU, Portland, Oregon, USA [a]
3',5'-T_2	formula., Berlin, Germany
3',5'-T_2AM	OHSU, Portland, Oregon, USA [a]
3'-T_1	formula., Berlin, Germany
3'-T_1AM	OHSU, Portland, Oregon, USA [a]
3-T_1	formula., Berlin, Germany
3-T_1AM	OHSU, Portland, Oregon, USA [a]
3-T_1AM-d4	OHSU, Portland, Oregon, USA [a]
acetic acid (glacial), 100 %, anhydrous	Merck, Darmstadt, Germany
acetone	J.T. Baker, Deventer, The Netherlands
acetonitrile	Merck, Darmstadt, Germany
AG 50W-X2 resin	Bio-Rad, Munich, Germany
agarose for electrophoresis	Serva, Heidelberg, Germany
albumin bovine fraction V, pH 7.0	Serva, Heidelberg, Germany
ammonium hydroxide solution, 1 M	Fluka, Steinheim, Germany
Bio-Rad IgG protein standard	Bio-Rad, Munich, Germany
Bio-Rad protein assay dye reagent	Bio-Rad, Munich, Germany
bromophenol blue	Roth, Karlsruhe, Germany

cyclohexane	Sigma-Aldrich, Steinheim, Germany
dATP solution, 100 mM	Fermentas, St. Leon-Rot, Germany
dCTP solution, 100 mM	Fermentas, St. Leon-Rot, Germany
ddH_2O	• Millipore (Darmstadt, Germany) for LC-MS/MS analyses • EASY pure UV compact ultrapure water system (Barnstedt, Germay) for all other experiments
DEPC	Roth, Karlsruhe, Germany
dGTP solution, 100 mM	Fermentas, St. Leon-Rot, Germany
dipotassium monohydrogen phosphate	Roth, Karlsruhe, Germany
disodium hydrogen phosphate	Merck, Darmstadt, Germany
DMSO	Sigma-Aldrich, Steinheim, Germany
DTT	Sigma-Aldrich, Steinheim, Germany
dTTP solution, 100 mM	Fermentas, St. Leon-Rot, Germany
EDTA	Sigma-Aldrich, Steinheim, Germany
ethanol	J.T.Baker, Deventer, The Netherlands
ethidium bromide solution, 10 mg/ml	Sigma-Aldrich, Steinheim, Germany
ethyl acetate	Sigma-Aldrich, Steinheim, Germany
formaldehyde solution, 37 %	J.T.Baker, Deventer, The Netherlands
formamide	AppliChem, Darmstadt, Germany
forskolin	Sigma-Aldrich, Steinheim, Germany
glycerol, 99 %	Sigma-Aldrich, Steinheim, Germany
HCl, 37 %	Merck, Darmstadt, Germany
HEPES	Merck, Darmstadt, Germany
K_2HPO_4	Merck, Darmstadt, Germany
KCl	Merck, Darmstadt, Germany
KH_2PO_4	Merck, Darmstadt, Germany
methanol	Roth, Karlsruhe, Germany
MG-132	Biomol, Plymouth, PA, USA
MOPS	Roth, Karlsruhe, Germany
$Na_2EDTA•H_2O$	Roth, Karlsruhe, Germany
$Na_2HPO_4•2H_2O$	Merck, Darmstadt, Germany
NaCl	Sigma-Aldrich, Steinheim, Germany

NaOH	Merck, Darmstadt, Germany
PMA	Sigma-Aldrich, Steinheim, Germany
PTU	MP Biomedicals, Eschwege, Germany
rT_3	formula., Berlin, Germany
rT_3AM	OHSU, Portland, Oregon, USA [a]
Sephadex™ LH-20	Amersham Biosciences, Piscataway, USA
sodium acetate	Roth, Karlsruhe, Germany
sodium selenite	Sigma-Aldrich, Steinheim, Germany
sucrose	Roth, Karlsruhe, Germany
T_0	formula., Berlin, Germany
T_0AM	OHSU, Portland, Oregon, USA [a]
T_4	formula., Berlin, Germany
T_4AM	formula., Berlin, Germany
TCA	Merck, Darmstadt, Germany
tris	Roth, Karlsruhe, Germany

[a] TAMs were generously provided by Dr. Thomas Scanlan, Department of Physiology & Pharmacology, Oregon Health & Science University, Portland, OR, 97239-3098, USA.

2.2 LC-MS/MS analysis

2.2.1 Equipment and consumables for LC-MS/MS analysis

TAM and TH analytes were separated using an Agilent 1100 integrated HPLC system consisting of a controller unit, two pumps including a degasser and an autosampler (Agilent, Waldbronn, Germany). Both the chromatographic column and the autosampler were operated at room temperature. Analytes were detected using an API365 triple quadrupole tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a TurboIonSpray interface. Nitrogen served as nebulizer, curtain and collision gas. The detection was performed using positive electrospray ionization (ESI+) in selected reaction monitoring (SRM) mode. Data processing was performed using Bio Analyst Version 1.3.1 software

(Applied Biosystems, Darmstadt, Germany). Other equipment and consumables used for the LC-MS/MS experiments are listed in Table 2.

Table 2: List of equipment and consumables used for LC-MS/MS analyses.

Product	**Supplier**
Analytical Guard Cartridge System (4.0 mm x 2.0 mm)	Phenomenex, Aschaffenburg, Germany
crimp caps with septum (Ø = 11 mm)	Wicom, Heppenheim, Germany
filters for HPLC eluent (pore size 0.45 µM)	Millipore, Bedford, USA
glass micro inserts (200 µl)	Wicom, Heppenheim, Germany
glass vials (2 ml, Ø = 11 mm)	Wicom, Heppenheim, Germany
Hamilton syringe device, H81320, 1001 TLL, 1 ml	Hamilton, Darmstadt, Germany
PEEK tubing (Ø = 0.13 mm)	Agilent, Waldbronn, Germany
scales “MC1 Analytic AC 210P”	Sartorius, Göttingen, Germany
syringe pump, Harvard Apparatus, #55-1111	Harvard Apparatus, Saint-Laurent, Canada
Synergi Polar-RP 80 Å column (150 mm x 2.0 mm)	Phenomenex, Aschaffenburg, Germany
ultrasonic bath, Sonorex Super RK 510H	Bandelin electronic GmbH, Berlin, Germany

2.2.2 Preparation of TAM and TH stock solutions

Two independently weighted and diluted 10 mM stock solutions were prepared for each TAM and TH analyte. Solvents were used as follows:

- The diiodothyronines and all TAMs except for T_4AM were dissolved in DMSO.
- T_0, monoiodothyronines, triiodothyronines and T_4AM were dissolved in DMSO containing 100 µM HCl.
- DMSO containing 100 µM NH_4^+ was used to dissolve T_4.

The solutions were stable at - 80 °C in darkness for at least 2 years (see sections 2.4.7 and 3.2.9).

2.2.3 Recording of mass spectra

The device-specific mass spectrometric parameters used to record the parent ion mass spectra and product ion tandem mass spectra are shown in Table 3.

Table 3: Device-specific mass spectrometric working parameters used to record the parent ion mass spectra and product ion tandem mass spectra.

Parameter	Value
NEB (valve position)	11
CUR (valve position)	10
CAD (valve position, for tandem mass spectra only)	5
TurboIon Spray (IS) voltage	5000 V
interface temperature	0 °C

The two 10 mM stock solutions that had independently been prepared for each analyte (section 2.2.2) were diluted in ddH_2O:acetonitrile:acetic acid (50:50:1) to obtain two individual 10 µM working solutions of each analyte. These working solutions were directly injected into the mass spectrometer at a flow rate of 10 µl/min using a Hamilton syringe device. To avoid background contaminations pure solvent was infused between the injections of different analyte working solutions. Both the parent ion mass spectrum and the product ion tandem mass spectrum of each 10 µM working solution were recorded 5 times on 5 different days within a period of 3 months.

2.2.4 Recording of chromatograms

The device-specific mass spectrometric parameters used to record the chromatograms are presented in Table 4.

Table 4: Device-specific mass spectrometric working parameters used to record the chromatograms.

Parameter	Value
flow rate of mobile phase	300 µl/min
NEB (valve postition)	11
CUR (valve position)	10
CAD (valve position)	5
TurboIon Spray (IS) voltage	5000 V
TurboIon Spray heater gas	8 l/min
interface temperature	300 °C
sample volume	10 µl
needle level	6 mm
sprayer position	+ 6 mm
dwell time	90 ms
Q1 peak width	0.7 amu
Q3 peak width	0.7 amu

Prior to each sample series the mobile phases were prepared freshly, filtered and degassed for 10 min using an ultrasonic bath. Furthermore, the column was equilibrated three times by performing three complete measurements injecting 10 µl mobile phase.

2.3 Extraction of TAMs and THs from Dio reactions

Dio reactions to be subjected to LC-MS/MS analysis were prepared as described in section 2.8.7. Enzymatic reactions were stopped by adding 0.1 volumes 100 % acetic acid. Subsequently, 20 nM 3-T_1AM-d4 were added to each vial to serve as internal standard (IS). Acidified reaction mixtures were incubated at 37 °C for 60 min. Proteins were precipitated by adding 3 volumes ice-cold acetone and incubating at - 20 °C for 15 min. After centrifugation at 14,000 x g and 4 °C for 5 min, supernatants were transferred into new Eppendorf tubes, acidified

with 0.002 volumes 30 % HCl, washed twice with 1 volume cyclohexane and then subjected to three subsequent extractions with 1.5 volumes ethyl acetate. Organic layers were combined and evaporated to dryness at 45 °C using a concentrator (eppendorf concentrator 5301, Eppendorf, Hamburg, Germany). Residues were redissolved in 30 µl ddH_2O:methanol:acetic acid (90:10:1) and stored at - 20 °C until analysis of deiodination products by LC-MS/MS. Injection volumes were 10 µl with analytes dissolved in the mobile phase used for LC-MS/MS analysis.

2.4 Validation of the LC-MS/MS method with extracted analytes

2.4.1 Selectivity, matrix effects, process efficiencies and recoveries

The following sets of samples were prepared to assess the selectivity of the LC-MS/MS method for TAM and TH analytes extracted from Dio reactions. Moreover, potential interfering effects of Dio reaction matrices with TAM and TH detection by LC-MS/MS as well as process efficiencies and recovery rates were determined.

- Samples of set 1 were Dio reactions used for LC-MS/MS analysis as described in Table 11 with the exception that no substrate but appropriate amounts of DMSO were added. Such blank samples were prepared of all types of Dio reactions used in this study, namely reactions containing lysates of HepG2, MSTO-211H, ECC-1, HEK293 cells or mouse liver membrane fractions either with or without 1 mM PTU. The respective Dio preparations had been heat-inactivated before. Dio reactions were subjected to liquid-liquid extraction as described in section 2.3.
- Samples of set 2 were obtained by adding 20 nM IS alone or together with 10 nM, 500 nM or 7.5 µM of each TAM and TH analyte to set 1 samples after liquid-liquid extraction.

- Samples of set 3 were prepared by adding 20 nM IS alone or together with 10 nM, 500 nM or 7.5 µM of each TAM and TH analyte to set 1 samples prior to liquid-liquid extraction.
- Both 3,3'-T_2AM and 3',5'-T_2AM as well as 3,3'-T_2 and 3',5'-T_2 were separated sufficiently to allow for an unequivocal qualitative identification of each analyte, but baseline peak separation was not achieved by the chromatographic method developed herein (section 3.1.2). Therefore, separate set 2 and set 3 samples were prepared containing only 3',5'-T_2AM and 3',5'-T_2.

For set 2 and set 3 samples, each analyte concentration was investigated with all types of Dio reactions. Each sample was prepared in triplicate. Samples were measured by LC-MS/MS and the mean values of the mass spectrometric response of the triplicate samples were determined. For set 2 and set 3 samples containing the IS alone, the absolute AUC of the IS was defined as the mass spectrometric response. By contrast, for samples containing the IS as well as TAM and TH analytes, the AUC ratio of analyte versus IS was considered as the mass spectrometric response as illustrated by the following formula:

$$\text{analyte AUC ratio} = \frac{\text{AUC of analyte}}{\text{AUC of 3-T}_1\text{AM-d4 (IS)}}$$

The selectivity of the LC-MS/MS method for TAM and TH analytes extracted from Dio reactions was investigated by comparing the mass spectrometer response of set 1 samples and set 2 samples within the analyte specific time windows.

Matrix effects were estimated by comparing the mass spectrometric response of set 1 samples to pure solvent (ddH_2O:acetonitrile:acetic acid, 50:50:1) and that of set 2 samples to solvent containing 20 nM IS alone or 20 nM IS together with the respective concentration of analytes. The following formula was used to report matrix effects in percent:

$$\text{matrix effect (\%)} = \frac{\text{mass spectrometric response of set 2 sample}}{\text{mass spectrometric response of the corresponding spiked solvent}} \text{ x 100 \%}$$

Hence, values below 100 % indicated analyte ion suppression whereas values above 100 % indicated analyte ion enhancement. The matrix effects for each analyte concentration were compared between the different deiodination reaction matrices using Kruskal-Wallis test.

Process efficiencies (a combination of matrix effects and recovery rates) were estimated by comparing the mass spectrometric response of set 3 samples to solvent containing 20 nM IS alone or 20 nM IS together with the respective concentration of analytes. The following formula was used to report process efficiencies in percent:

$$\text{process efficiency (\%)} = \frac{\text{mass spectrometric response of set 3 sample}}{\text{mass spectrometric response of the corresponding spiked solvent}} \times 100\ \%$$

The process efficiencies of each analyte concentration were compared between the different deiodination reaction matrices using Kruskal-Wallis test.

Recoveries were determined by comparing the mass spectrometric response of set 2 and set 3 samples. Values were calculated in percent using the following formula:

$$\text{recovery rate (\%)} = \frac{\text{mass spectrometric response of set 3 sample}}{\text{mass spectrometric response of the corresponding set 2 sample}} \times 100\ \%$$

The recoveries of each analyte concentration were compared between the different deiodination reaction matrices using Kruskal-Wallis test.

For recoveries, this series of experiments was repeated twice within 1 week. Thus, for each analyte and for each matrix, three grand means were obtained, which were again compared using Kruskal-Wallis test. These means served as reference values since they were compared to the recoveries determined in each routine series of samples described herein. Any routine series of samples was accepted when the analyte specific recoveries were within the 15 % coefficient of variation (CV) interval of the three analyte specific grand means.

2.4.2 Linearity and validation of 3-T_1AM-d4 as an internal standard

The following series of experiments was performed to determine the linear ranges of the LC-MS/MS method with extracted TAM and TH analytes. Furthermore, 3-T_1AM-d4 should be validated as IS for all analytes. For this purpose, Dio reactions were prepared as described for use in LC-MS/MS analysis in Table 11 using heat-inactivated cell line lysates or mouse liver membrane fractions. DMSO or TAM and TH analytes were added yielding the following samples:

- Samples of set 1 contained all TAMs and THs except for 3',5'-T_2AM and 3',5'-T_2 at final concentrations of 250 pM, 1 nM, 5 nM, 25 nM, 100 nM, 250 nM, 500 nM, 1 µM, 2.5 µM, 5 µM and 7.5 µM.
- Samples of set 2 contained 3',5'-T_2AM and 3',5'-T_2 at the same final concentrations. The reasons for excluding 3',5'-T_2AM and 3',5'-T_2 from these samples are described in section 3.1.2.
- Samples of set 3 contained an appropriate amount of DMSO to serve as blank control.

The IS was added to each sample at a constant concentration of 20 nM. All three sets of samples were prepared for all Dio reaction matrices. Samples of set 1 and set 2 were prepared in six replicates except for the samples containing 250 pM and 7.5 µM of analytes, which were prepared in ten replicates. Set 3 samples were also prepared in ten replicates. Samples were subjected to liquid-liquid extraction (section 2.3) and measured by LC-MS/MS. At each concentration outliers were identified using Grubbs test and eliminated if found to be significant ($p < 0.05$). To check for homogeneity of variance over the calibration range, the variances of the samples containing TAM and TH analytes at final concentrations of 250 pM and 7.5 µM were compared using F-test. For all analytes and all Dio reaction matrices, homogeneity of variances was achieved using a weighted (1/analyte concentration) least squares model, which was therefore used to calculate the calibration curves. The slopes and y-intercepts of the analyte specific

regression lines were compared between the different Dio reaction matrices using Kruskal-Wallis test.

This series of experiments was repeated twice within 1 week. Thus, for each analyte and for each Dio reaction matrix, three calibration curves were obtained. These curves served as reference curves since they were compared to the calibration curves determined in each routine series of samples described herein. Any routine calibration curve was accepted if its slope and y-intercept were within the 15 % coefficient of variation interval of the three reference curves.

2.4.3 Limit of detection and limit of quantification with extracted analytes

Set 1 and set 2 samples were prepared as described for calibration curve experiments except that the following final analyte concentrations were achieved in the Dio reaction matrices: 100 pM, 250 pM, 500 pM, 750 pM, 1 nM, 2.5 nM, 5.0 nM 7.5 nM, 10.0 nM and 12.5 nM. Each sample was measured by LC-MS/MS. From the set 3 samples which had been generated for calibration curve experiments (section 2.4.2), the signal-to-noise ratios were calculated within the time windows in which the analytes were expected. The lowest analyte concentration yielding an AUC ratio of at least 3 times the signal-to-noise ratio was defined as the limit of detection (LOD). The lowest analyte concentration yielding an AUC ratio of 10 times the signal-to-noise ratio was defined as the limit of quantification (LOQ). This sample series was repeated twice.

2.4.4 *Intra-assay* precision and bias

To validate the analyte specific *intra-assay* precisions and bias of the LC-MS/MS method for extract TAM and TH analytes, set 1 and set 2 samples were prepared as described for calibration curve experiments except that the analytes were added at final concentrations of 10 nM, 500 nM and 7.5 µM. Each sample was measured 10 times consecutively in the same run. Analyte concentrations were

calculated using the routine calibration curves. For each analyte, the *intra-assay* precision of the retention time was calculated as standard deviation while the *intra-assay* precision of the analyte AUC concentration was determined as the coefficient of variation. The *intra-assay* bias of the analyte concentrations were calculated as the percent deviations of the observed mean values from the respective reference values according to the following formula:

$$\text{bias } (\%) = \frac{x - \mu}{\mu} \times 100\ \%$$

x	observed analyte concentration
μ	reference analyte concentration

The analyte specific values for *intra-assay* precision and bias were compared between the different analyte concentrations and the different deiodination reaction matrices using Kruskal-Wallis test.

2.4.5 *Inter-assay* precision and bias

Thirty aliquots of each of the aforementioned samples that had been generated to determine the *intra-assay* precision and bias, were stored in darkness at - 80 °C. Three aliquots of each sample were measured on 10 different days over a time period of 2 months. The concentrations of the analytes were calculated using the routine calibration curves. The triplicate values conducted for each analyte retention time and each analyte concentration obtained from every series of experiments were used to calculate a daily mean. For each analyte, the *inter-assay* precision of the retention time was calculated as the standard deviation of the daily means subtracted by the analyte specific *intra-assay* precision. By contrast, the *inter-assay* precision of the analyte concentration was determined as the coefficient of variation of the daily means subtracted by the respective *intra-assay* precision. The *inter-assay* bias was calculated as the percent deviation of the grand means from each daily series of experiments from the respective nominal

value. The analyte specific values for *inter-assay* precision and bias were compared between the different analyte concentrations and the different deiodination reaction matrices using Kruskal-Wallis test.

2.4.6 Analyte stability in extracted Dio assays

The following experiments were performed in order to control the stability of each individual TAM and TH analyte under auto-sampler and storing conditions, which included monitoring for non-enzymatic deiodinations. For that purpose, Dio reactions were prepared as described for use in LC-MS/MS analysis in Table 11 using heat-inactivated cell line lysates or mouse liver membrane fractions. Each TAM and TH analyte was added individually at final concentrations of 10 nM, 500 nM and 7.5 µM while the IS was added at a constant final concentration of 20 nM to all samples. Six replicates of each sample were prepared in every type of Dio reaction matrix. Samples were subjected to liquid-liquid extraction and the residues were aliquoted. To monitor auto-sampler stability, the aliquots of freshly prepared residues of three replicate samples were measured at the beginning, in the middle and at the end of a 2 day LC-MS/MS run. To assess storage stability, the aliquots of residues of three replicate samples were measured directly on the day of sample preparation and compared to aliquots which had been stored at - 20 °C for 1, 2 or 3 days, which was the longest storing period used in this study. The coefficient of variation of the analyte AUC ratio was calculated for every analyte concentration and Dio reaction matrix. The values obtained for each analyte concentration level and each deiodination reaction matrix were compared using Kruskal-Wallis test.

2.4.7 Long-term stability of TAM and TH stock solutions

From the 10 mM TAM and TH stock solutions prepared as described in section 2.2.2 the following two samples were prepared using DMSO as solvent:

- Sample 1 contained all TAM and TH analytes except for 3',5'-T_2AM and 3',5'-T_2 at a final concentration of 100 µM. The reasons for excluding 3',5'-T_2AM and 3',5'-T_2 from these samples are described in section 3.1.2.
- Sample 2 contained 3',5'-T_2AM and 3',5'-T_2 at a final concentration of 100 µM.

The IS was added to every sample at a final constant concentration of 20 µM. Twenty aliquots of both samples were stored in darkness at - 80 °C, - 20 °C and 4 °C, respectively. At the beginning of each routine run, after equilibration of the column, 1 aliquot of sample 1 and 2 from each storing condition was diluted 1:1000 in solvent (ddH_2O:acetonitrile:acetic acid, 50:50:1) and subjected to LC-MS/MS measurement. To compare the analyte AUC ratios, the coefficients of variations between the storing temperatures were calculated. These experiments were performed over a period of 2 years.

2.5 Animal organs

Livers from two male, adult, euthyroid C57BL/6 wild type mice at 24 weeks of age were kindly provided by Dr. Ulrich Schweizer (Institute of Experimental Endocrinology, Charité - Universitätsmedizin, Berlin, Germany).

2.6 Cell culture

2.6.1 Cell lines, cell culture media and consumables

Tables 5 and 6 list the human cell lines, cell culture media and consumables used in this study.

Table 5: List of human cell lines serving as isozyme specific sources of Dios.

Designation	Organism	Tissue	Origin: ATCC® number [a]
ECC-1	human	endometrium carcinoma	CRL-2923
HEK293	human	human embryonic kidney	CRL-1573
HepG2	human	hepatocarcinoma	HB-8065
MSTO-211H	human	mesothelioma	CRL-2081

[a] identification number provided by the American Type Culture Collection (ATCC)

Table 6: List of cell culture media and consumables used in this study.

Product	Supplier
cell culture flasks (25, 75, 150 cm^2)	Biochrom, Berlin, Germany
cell scraper, 30 cm	Biochrom, Berlin, Germany
DMEM medium	Biochrom, Berlin, Germany
DMEM-F12 medium	Invitrogen, Karlsruhe, Germany
falcon tubes (15, 50 ml)	Biochrom, Berlin, Germany
FCS	Biochrom, Berlin, Germany
RPMI-1640 medium	Invitrogen, Karlsruhe, Germany
serological pipettes (1, 2, 5, 10, 25 ml)	Biochrom, Berlin, Germany
6-well plates	Biochrom, Berlin, Germany
trypsin/EDTA solution in PBS w/o Ca^{2+}, Mg^{2+}	Biochrom, Berlin, Germany

2.6.2 Routine propagation of cell lines

HepG2 cells and ECC-1 cells were propagated in DMEM-F12 medium. HEK293 cells were cultured in DMEM medium while MSTO-211H cells were grown in RPMI-1640 medium. All cell culture media were supplemented with 10 % (v/v) FCS and 100 nM sodium selenite to allow for optimal expression of Dio seleno-proteins. Addition of antibiotics to the cell culture medium was omitted since some antibiotics, such as aminoglycosides, have been shown to induce stop-codon read-through and concomitant amino acid substitution at the selenocysteine (Sec)

codon in mammalian cells, which in turn led to a decrease in selenoenzyme activity, as Sec is essential for normal catalytic center function in selenoenzymes, such as Dio isozymes [Handy, 06]. Cells were propagated in an incubator (Hera cell incubator, Heraeus, Hanau, Germany) at 37 °C, 95 % humidity and at 5 % CO_2.

Cells were passaged at a confluence of 80 to 90 % under sterile conditions (Lamin Air® HB2448, Heraeus Instruments, Hanau, Germany). After rinsing twice with 1 x PBS, cells were detached by incubation in trypsin/EDTA solution for 3 min at standard growing conditions. Trypsin was inhibited by adding fresh medium containing 10 % (v/v) FCS. Cells were centrifuged for 5 min at 1000 x g and 20 °C. After removing the supernatant, cells were resuspended in fresh medium and seeded at a minimum confluence of 20 %.

2.6.3 Stimulation of specific Dio2 and Dio3 enzymatic activity

Confluent MSTO-211H cells were serum-starved over night and then incubated in the presence of 10 µM forskolin and 700 nM MG-132 for 6 h in order to stimulate the endogenous Dio2 enzymatic activity [Curcio, 01]. Likewise, confluent and serum-starved ECC-1 cells were treated with 100 nM PMA for 6 h in order to increase the endogenous Dio3 enzymatic activity [Kester, 06].

2.7 Gene expression analysis

2.7.1 Working solutions for RNA analysis

Table 7 lists the working solutions prepared for gene expression analysis. Unless otherwise stated, all working solutions were stored at room temperature.

Table 7: Composition of working solutions prepared for analysis of gene expression.

Designation	Composition
1 x MOPS buffer	diluted from 10 x MOPS buffer using ddH_2O
10 x MOPS buffer	4.0 M MOPS at pH 7.0, 1.0 M sodium acetate, 0.1 M EDTA in ddH_2O, pH 7.0, autoclaved, stored in darkness
1 x TAE buffer	40 mM tris-acetate, 1 mM EDTA in ddH_2O, pH 8.3
5 x RNA loading buffer	4 mM EDTA, 720 µl 37 % (v/v) formaldehyde solution, 2 ml 100 %(v/v) glycerol, 3084 µl formamide, 4 ml 10 x MOPS, 16 µl saturated aqueous bromophenol blue solution, ad 10 ml DEPC water, stored at 4 °C
6 x DNA loading buffer	0.25 % (v/v) saturated, aqueous bromphenol blue solution, 30 % (v/v) glycerol in ddH_2O, stored at 4 °C
DEPC water	1 ‰ DEPC was added to ddH_2O, incubated at room temperature for 24 h, sterilized by autoclaving and stored at - 20 °C.
dNTP-Mix (100 mM)	equal volumes of 100 mM solutions of dATP, dCTP, dGTP, dTTP, stored at - 20 °C
molecular size marker	1 µl Gene Ruler™ 1 kb DNA ladder plus (Fermentas, St. Leon-Rot, Germany), 1 µl 6 x loading dye solution (Fermentas, St. Leon-Rot, Germany), 4 µl ddH_2O

2.7.2 RNA isolation from human cell lines and mouse liver

RNA was isolated from $5 \cdot 10^5$ cells and from pulverized mouse livers using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacture's instructions.

2.7.3 Determination of RNA concentration and purity

To determine the concentrations and purities of RNA samples, 1 µl of each sample was applied directly to the NanoDrop® ND-1000 Spectrophotometer (peqlab, Erlangen, Germany). The UV/Vis spectra were recorded from 220 nm to 750 nm using DEPC water as blank control. Samples were measured in duplicates. The RNA concentrations were calculated applying the optical density at 260 nm and a molar absorption coefficient of 40 ng/µl. The ratios of OD_{260} to OD_{280} served to estimate RNA purities and were at least 2.0.

2.7.4 Control of RNA quality by gel electrophoresis and densitometry

RNA quality was monitored by horizontal denaturating agarose gel electrophoresis. Agarose was dissolved by boiling in DEPC water. Prior to solidification of the gel, 10 % (v/v) formaldehyde solution (37 %), 10 % (v/v) 10 x MOPS buffer and 5 µl ethidium bromide solution/100 ml gel were added. For sample preparation, 2 µg total RNA and 2 µl 5 x RNA loading buffer were adjusted to a final volume of 10 µl by adding DEPC water. Samples were heat denaturated at 65 °C for 5 min and subsequently cooled down on ice for 2 min. 9 µl of each sample were loaded onto the gel. Electrophoresis was performed in a horizontal Blue Marine 200 electrophoresis unit (Serva, Heidelberg, Germany) for 2 h applying 4 V/cm gel and using 1 x MOPS buffer as running buffer.

After electrophoresis, gels were scanned using the Vilber Lourmat gel documentation system (LTF Labortechnik, Wasserburg, Germany). Densitometric analyses were performed using the BioCapt software. Optically well-defined bands of 28 S rRNA and 18 S rRNA whose background corrected densitometric intensities on the scanned gel displayed a ratio at least 1.9 were considered indicative of intact total RNA.

2.7.5 cDNA synthesis by RT- PCR

cDNA was synthesized from 5 µg total RNA using the RevertAid^{TM} first strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany) according to the manufacture's instructions. Incubations were performed in a thermal cycler (Primus, MWG Biotech, Ebersburg, Germany).

2.7.6 Semiquantitative PCR

PCR reactions were performed using the Taq DNA polymerase kit (Qiagen, Hilden, Germany) according to the manufacture's instructions. Gene specific primer pairs were designed on the basis of cDNA sequences deposited in the GenBank

database. The primer pairs used in each reaction, their nucleotide sequences as well as the respective PCR product sizes are listed in Table 8.

Table 8: List of gene-specific primer pairs used for PCR analyses including the respective nucleotide sequences and PCR product sizes.

Target gene	Notation of primers	GenBank Accession	Nucleotide sequences of primers (5' to 3')	Product size (bp)
hDio1	hDio1 fwd	NM000792	GTGCATGTGGTCGTGGGTAAA	604
	hDio1 rev		CCCTCCTGGATTATGTAGAGCCTC	
hDio2	hDio2 fwd	NM000793	TTCCTGCTGGTCTACATT	796
	hDio2 rev		GGGAATTTTCCAACTGGG	
hDio3	hDio3 fwd	NM001362	ATCATCTACATCGAGGAA	942
	hDio3 rev		GAGATAGTTCGTCATCAG	
hGAPDH	hGAPDH fwd	NM002046	CCACCCATGGCAAATTCCATGGCA	592
	hGAPDH rev		TCTAGACGGCAGCTCAGGTCCACC	
mDio1	mDio1 fwd	NM007860	TCCAGTACTTCTGGTTTGTCCT	488
	mDio1 rev		CAGCTTTACCCTTGTAGCAGAT	
mDio2	mDio2 fwd	NM010050	ACTTCTCTACCACCACCTTCCT	384
	mDio2 rev		CGAGGCATAATTGTTACCTGAT	
mDio3	mDio3 fwd	NM172119	ACAGATTCACCCTATGTCATCC	378
	mDio3 rev		CAACCTTTGACTTTCTTTGGAG	
mGAPDH	mGAPDH fwd	NM008084	TGGCAAAGTGGAGATTGTTGCC	155
	mGAPDH rev		AAGATGGTGATGGGCTTCCCG	

The composition of the PCR samples was optimized in preliminary experiments to achieve sensitive amplification of single PCR products. According to the optimized protocol, each PCR sample contained 1 µl undiluted cDNA as template, 5 µl 10 x PCR buffer, 1 mM $MgCl_2$, 250 mM dNTP mix, 100 nM respective fwd primer, 100 nM respective rev primer, 1.25 U Taq polymerase and was adjusted to a final volume of 50 µl by adding 40.25 µl DEPC water. The specificity of each PCR was confirmed in every experiment by incubating one sample in the absence of template cDNA.

The PCR samples were incubated in a thermal cycler (Primus, MWG Biotech, Ebersburg, Germany). In preliminary experiments, annealing temperatures between 58 °C and 65 °C were tested for each gene in order to optimize the sensitivity, specificity and efficiency of the reactions. In subsequent optimization experiments, the log-linear range of each PCR was determined by starting several identical reactions and removing the tubes one by one from the cycler after every second reaction cycle. For the hGAPDH and mGAPDH gene, a cycle number within the log-linear range was chosen. By contrast, for the Dio genes, a cycle number beyond the log-linear range was chosen since it was more important to exclude the expression of individual Dio transcripts in a cell line or tissue than to semiquantify the concentrations of the detectable Dio mRNAs. The optimized cycler conditions are presented in Table 9.

Table 9: Optimized thermal cycler conditions for PCR analyses.

Step	Reaction	Temperature (°C)	Incubation time
1	denaturation	95	3 min
2	annealing	optimized: hDio1: 64, hDio2: 58, hDio3: 58, hGAPDH: 61 mDio1, mDio2, mDio3, mGAPDH: 58	1 min
3	elongation	72	1 min
4	denaturation	95	30 s
5	annealing	optimized, see above	40 s
6	elongation	72	1 min
7	loop to step 4	optimized cycle numbers: h/mDio1, h/mDio2, h/mDio3: 40, h/mGAPDH: 21	
8	final elongation	72	5 min

2.7.7 Gel electrophoresis of PCR products

The sizes of the PCR products were estimated by horizontal agarose gel electrophoresis. Agarose was dissolved by boiling in 1 x TAE buffer. Appropriate agarose concentrations were chosen to allow for sufficient separation of dsDNA in the size range of the respective PCR product, namely 2.0 % (w/v) for 100 to 500 bp and

1.5 % (w/v) for 500 to 1,000 bp. Prior to the solidification of the gel, 5 µl ethidium bromide solution/100 ml gel were added. For sample preparation, 10 µl PCR product and 3 µl 6 x DNA loading buffer were adjusted to a final volume of 18 µl by adding 5 µl ddH_2O. 15 µl of each sample were loaded onto the gel. To estimate the sizes of the PCR products, 5 µl molecular size marker were also electrophorized on each gel. Electrophoresis was performed in a horizontal Blue Marine 200 electrophoresis unit (Serva, Heidelberg, Germany) for 45 min applying 6 V/cm gel and using 1 x TAE buffer as running buffer. After electrophoresis, gels were scanned using the Vilber Lourmat gel documentation system (LTF Labortechnik, Wasserburg, Germany).

2.8 Deiodinase assays

Two types of Dio assays were used in this study. The classical assay, which was based on the release of radioiodide from a tracer, such as [5'-^{125}I]rT_3, was developed by Leonard and Rosenberg in 1980 and will be referred to as "$^{125}I^-$ release assay". It allowed for quantifying specific Dio1 and Dio2 activites. The novel assay, which relied on the measurement of deiodinated TAM or TH substrate and product by LC-MS/MS, was developed in this study and will be referred to as "LC-MS/MS based Dio assay". This method was suited to measure specific Dio1, Dio2 and Dio3 activities.

2.8.1 Working solutions for Dio assays

The working solutions prepared for use in Dio assays are listed in Table 10.

Table 10: Working solutions prepared for Dio assays.

Working solution	Composition
1 mM DTT	1 mM DTT in ddH_2O, stored at - 20 °C
1 x PBS	137 mM NaCl, 10 mM $Na_2HPO_4 \cdot H_2O$, 2.6 mM KCl, 1.8 mM KH_2PO_4, ad 1000 ml ddH_2O, pH 7.4, autoclaved, stored at 4 °C
10 % (v/v) acetic acid	10 % (v/v) acetic acid, stored at room temperature
10 % (w/v) TCA	10 % (w/v) TCA in ddH_2O, stored at 4 °C
10 mM PTU	PTU was mixed with 40 mM NaOH. 100 mM NaOH was added dropwise until PTU dissolved. pH was < 9.0. Stored at - 20 °C.
100 mM HCl	100 mM HCl in ddH_2O, stored at room temperature
40 mM NaOH	40 mM NaOH in ddH_2O, stored at room temperature
AG 50W-X2 resin columns	3 volumes AG 50W-X2 were stirred with 2 volumes 10 % acetic acid and filled into sarpette tips (2 ml PS, Sarstedt, Nümbrecht, Germany) equipped with filters (10 µm pore sized, MoBiTec, Göttingen, Germany). Stored at room temperature.
BioRad protein dye working solution	Bio-Rad protein assay dye reagent was diluted 1:5 in ddH_2O immediately before use.
Dio homogenization buffer	250 mM D(+) sucrose, 20 mM HEPES, 1 mM EDTA in ddH_2O, pH 7.4 or pH 8.0, stored at 4 °C, 1 mM DTT was added prior to usage.
eluent	ethanol:1 M ammonium hydroxide solution (49:1), stored at room temperature
potassium-phosphate-EDTA-buffer	Buffer A (1 M K_2HPO_4, 10 mM $Na_2EDTA \cdot 2H_2O$ in ddH_2O) was titrated with buffer B (1 M KH_2PO_4, 10 mM $Na_2EDTA \cdot H_2O$ in ddH_2O) to adjust pH 6.8 or pH 8.0. Stored at 4 °C.
protein standard	Bio-Rad IgG protein standard was reconstituted according to the manufacture's instruction, aliquoted and stored at - 20 °C.
Sephadex LH-20	20 % (w/v) Sephadex LH-20 in 100 mM HCl, stored at room temperature
stopping solution	10 % albumin bovine fraction V, 10 mM PTU in ddH_2O, stored at - 20 °C

2.8.2 Optimization of sample preparation and reaction conditions for Dio assays

The Dio isozymes differ considerably in their kinetic parameters and substrate preferences [Köhrle, 02]. Therefore, sample preparation and reaction conditions were optimized for each isozyme in preliminary experiments. The optimized protocols are listed in Table 11.

In the present study, two independent methods were used to analyze Dio catalyzed reactions, Firstly, classical $^{125}I^-$ release assays were employed, which quantify the release of $^{125}I^-$ from radiolabeled TH substrates [Leonard, 80]. Secondly, the LC-MS/MS method and the liquid-liquid extraction protocol developed herein were used to study the Dio catalyzed conversions of non radioactively labeled TAM and TH substrates. In $^{125}I^-$ release assays and in LC-MS/MS based Dio assays, which were designed to measure specific Dio activities and kinetic parameters of Dio catalyzed reactions, the reaction conditions were optimized to ensure that the reaction rates were proportional to the concentrations of TAM or TH substrate, cofactor DTT and protein as well as to the incubation time (Figures 10, 12, 14, 16, 18, 24; Tables 18, 19). In LC-MS/MS experiments which aimed at screening the substrate specificity of the Dio isozymes towards TAM and TH substrates, a prolonged incubation time of 120 min was used (Figures 11, 13, 15, 17, 19 - 22).

Table 11: Optimized protocols for sample preparations and incubation conditions for Dio assays.

Source of Dio preparation	**HepG2**		**mouse liver**		**MSTO-211H**		**ECC-1**		**HEK293**	
type of Dio assay	$^{125}I^-$ [a]	LC-MS/MS	$^{125}I^-$ [a]	LC-MS/MS	$^{125}I^-$ [a]	LC-MS/MS	$^{125}I^-$ [a]	LC-MS/MS	$^{125}I^-$ [a]	LC-MS/MS
pH of homogenization buffer	7.4	7.4	7.4	7.4	7.4	7.4	8.0	8.0	7.4 or 8.0 [b]	7.4 or 8.0 [b]
µg protein/reaction	50	300	20	40	150	2000	300	600	20-600 [b]	40-2000 [b]
[substrate] (µM)	0.05-2.0	0.05-20.0	0.2-4.4	0.05-20.0	0.002-0.05	0.01-0.5	1.0	0.05-5.0	0.01-4.4 [b]	0.01-20 [b]
pH of potassium-phosphate-EDTA buffer	6.8	6.8	6.8	6.8	6.8	6.8	8.0	8.0	6.8 or 8.0 [b]	6.8 or 8.0 [b]
[cosubstrate (DTT)] (mM)	20	20	20	20	20	20	50	50	20 or 50 [b]	20 or 50 [b]
incubation time (min)	30	30 [c] or 120 [d]	15	15 [c] or 120 [d]	30	30 [c] or 120 [d]	30	30 [c] or 120 [d]	15-30 [b]	15-120 [b]

[a] $^{125}I^-$ release assay
[b] adapted to the conditions used in the respective series of samples
[c] as used in quantitative LC-MS/MS based Dio assays which were designed to measure specific Dio activities and kinetic parameters of Dio catalyzed reactions
[d] as used in qualitative LC-MS/MS based Dio assays which were designed to screen the substrate specificity of Dio isozymes towards TAM and TH substrates

2.8.3 Generation of cell line derived Dio preparations

Dio1 containing preparations were generated from untreated HepG2 cells. By contrast, Dio2 and Dio3 containing preparations were obtained from MSTO-211H and ECC-1 cells, respectively which had been pretreated as described in section 2.6.3 to enhance the endogenous Dio2 or Dio3 expression. Dio deficient control preparations were obtained from untreated HEK293 cells.

Cell layers were washed twice with ice-cold 1 x PBS and harvested by scraping in Dio homogenization buffer containing 1 mM freshly added DTT. HepG2 cells and MSTO-211H cells were harvested in homogenization buffer at pH 7.4 whereas ECC-1 cells were scraped in homogenization buffer at pH 8.0 (Tables 10, 11). Cell lysates were frozen in liquid nitrogen, thawed on ice and disrupted by sonication (ultrasonic processor, Braun, Melsungen, Germany) applying ten pulses of 0.6 s at 100 w. Protein concentrations were determined as described in section 2.8.5. The protein concentration required for either $^{125}I^-$ release assays or LC-MS/MS based Dio assays was adjusted using the respective Dio homogenization buffer (Table 11). The Dio preparations were aliquoted into 40 µl samples for use in $^{125}I^-$ release assays and 80 µl samples for use in LC-MS/MS based Dio assays. Samples were stored at - 20 °C until use.

2.8.4 Preparation of mouse liver membrane fractions

For preparation of Dio1 containing mouse liver membrane fractions, livers from adult male C57BL/6 mice were pulverized (micro-dismembrator U, Braun, Melsungen, Germany). Tissue powder was homogenized in Dio homogenisation buffer at pH 7.4 containing 1 mM DTT using a 1 ml luer-lock syringe (BD Plastipak Becton Dickinson, Heidelberg, Germany) equipped with a disposable insulin needle (0.45 x 12 mm, Braun, Melsungen, Germany) and sonication as described above. Homogenates were centrifuged at 10,000 x g and 4 °C for 10 min (Centrifuge 54 17 R, Eppendorf, Hamburg, Germany). Supernatants were discarded. Pellets were resuspended in Dio homogenisation buffer at pH 7.4 and subjected

to another course of sonication. Protein concentrations were measured as described in section 2.8.5. Appropriate protein concentrations were adjusted using Dio homogenization buffer at a pH of 7.4. The mouse liver membrane fractions were aliquoted into 40 µl samples for use in $^{125}I^-$ release assays and 80 µl samples for use in LC-MS/MS based Dio assays. Samples were stored at - 20 °C until use.

2.8.5 Determination of protein concentration of Dio preparations

The protein concentration of Dio preparations, namely cell line lysates and mouse liver membrane fractions, was determined by a modified Bradford protein assay [Bradford, 76]. For sample preparation, an appropriate amount of cell line lysate or mouse liver membrane fractions (usually 1 to 5 µl) was adjusted to 100 µl using ddH_2O. Each sample was assessed in triplicates. In standard curve samples 1, 2, 4, 5, 6 and 8 µl of protein standard (the IgG concentrations varied between batches ranging from 1.34 µg/µl to 1.45 µg/µl) were also adjusted to 100 µl using ddH_2O. The blank value contained 100 µl ddH_2O. Samples were incubated with 900 µl BioRad protein dye working solution for 5 min at room temperature and then transferred into 1.5 ml curettes (Sestet, Numbest, Germany). OD_{595} values were measured in an Eppendorf Bio Photometer (Eppendorf, Hamburg, Germany). The OD_{595} values of the standard curve samples were plotted against their protein concentrations. Linear regression analysis typically yielded standard curves with coefficients of determination (r^2) > 0.95 allowing for calculation of protein concentrations of Dio preparations.

2.8.6 $^{125}I^-$ release assays

The 40 µl aliquots of Dio preparations (see sections 2.8.3 and 2.8.4) were thawed on ice. Each sample was assessed using six replicate aliquots. Three replicates were mixed with 10 µl ddH_2O and 10 µl 10 mM PTU, respectively. A substrate buffer was prepared according to the master mix protocol listed in Table 12.

Table 12: Composition of the substrate buffer master mix used in $^{125}I^-$ release assays.

Reagent	Volume/aliquot
potassium-phosphate-EDTA-buffer, pH 7.4	10 µl
40 mM NaOH	0.41 µl
1 M DTT	2 µl
unlabeled rT_3 (for concentrations see Table 11)	0.1 µl
ddH_2O	37.49 µl

Prior to each assay, [5'-^{125}I]rT_3 was prepurified from $^{125}I^-$ originating from autoradiolytic decay. [5'-^{125}I]rT_3 was applied to a freshly prepared column consisting of 600 µl hydrochloric acid-equilibrated Sephadex LH-20 and a 10 µm pore sized filter (MoBiTec, Göttingen, Germany). $^{125}I^-$ was eluted by washing the column with 3 ml 100 mM HCl. After equilibrating the column with 3 ml ddH_2O, [5'-^{125}I]rT_3 was eluted into a fresh borosilicate glass tube (16 x 100 mm, Hartenstein, Würzburg, Germany) by applying 1 ml ethanol:1 M ammonium hydroxide solution (49:1) to the column and subsequent centrifugation for 5 min at 1,000 x g and 4 °C (Cryofuge 5000, Heraeus Sepatech, Hanau, Germany). The tube was placed into a 50 °C water bath and the eluent was evaporated under a stream of nitrogen. The dried [5'-^{125}I]rT_3 was dissolved directly in substrate buffer. To quantify the radioactivity of [5'-^{125}I]rT_3 in the substrate mix, the volume of the substrate mix to be applied to a single sample was mixed with 2 ml 10 % (v/v) acetic acid. Three replicates were prepared and measured in a γ-counter (1277 Gammamaster, LKB Wallac, Turku, Finland). The radioactivity of [5'-^{125}I]rT_3 in the substrate mix was adjusted to 1,000 ± 200 cpm/µl.

Deiodination reactions were started by adding 50 µl substrate buffer to each tube and immediately placing the tubes one by one into a water bath at 37 °C. To correct for background $^{125}I^-$ release which was not catalyzed by Dios, three blank samples devoid of any Dio preparation were included at the beginning and at the end of the incubation of each series of samples. To monitor *inter-assay* variations, aliquots from an in-house Dio1 preparation originating from mouse liver were also

included. For all $^{125}I^-$ release assays carried out in this study, the specific Dio1 activity measured in these samples were within a 15 % coefficient of variation (CV) interval.

Deiodination reactions were stopped by adding 0.5 volumes stopping solution to bind iodothyronines and by placing the samples on ice. 40 µl Dio preparations were added to the six blank samples. Three volumes 10 % (w/v) TCA were added and mixed with samples to precipitate proteins. After centrifugation at 14,000 x g for 5 min at room temperature (Centrifuge 5415C, Eppendorf, Hamburg, Germany) iodothyronines, including [5'-^{125}I]rT_3, were contained in the protein pellets whereas released I^-, including $^{125}I^-$, was contained in the supernatants. To definitely separate $^{125}I^-$ from [5'-^{125}I]rT_3, 480 µl of each supernatant were applied to an AG 50W-X2 resin column, which had been equilibrated with 5 ml 10 % acetic acid. Released I^- was eluted into polystyrene tubes (5 ml, Sestet, Numbest, Germany) by applying 2 ml 10 % (v/v) acetic acid to each column. Released $^{125}I^-$ was quantified in the γ-counter.

Specific Dio1 and Dio2 activities were calculated according to the following formula:

$$\text{specific Dio activity (pmol } I^- \text{ released/mg/min)} = \frac{2 \bullet (cpm_{sample} - cpm_{blank}) \bullet n_{rT3} \bullet 1000}{cpm_{100\%} \bullet m_{protein} \bullet t \bullet 480/550}$$

2	factor accounting for that each released $^{125}I^-$ reflects only half of the 5'-deiodinations of 5'[^{125}I]rT_3
cpm_{sample}	mean radioactivity of triplicate aliquots of each sample (cpm)
cpm_{blank}	mean radioactivity of six blank samples (cpm)
n_{rT3}	amount of unlabeled rT_3 per reaction (nmol)
$cpm_{100\ \%}$	mean of radioactivity in substrate mix per sample (cpm)
$m_{protein}$	amount of protein per reaction (mg)
t	incubation time (min)
480/550	factor accounting for analyzing 480 µl of the 550 µl supernatants

The $^{125}I^-$ released in samples containing PTU was attributed to Dio2 while the difference in $^{125}I^-$ release between aliquots with and without PTU was used to calculate Dio1 activity.

2.8.7 Dio assays based on LC-MS/MS

The 80 µl aliquots of Dio preparations (see sections 2.8.3 and 2.8.4) were thawed on ice. In line with $^{125}I^-$ release assays, each sample was assessed using six replicate aliquots. Three replicates were mixed with 20 µl ddH_2O and 20 µl 10 mM PTU, respectively. A substrate buffer was prepared according to the master mix protocol listed in Table 13.

Table 13: Composition of the substrate buffer master mix used in LC-MS/MS based Dio assays.

Reagent	**Volume/aliquot**
potassium-phosphate-EDTA-buffer (for pH see Table 11)	20 µl
40 mM NaOH	0.82 µl
1 M DTT	4 µl or 10 µl (see Table 11)
TAM or TH standard solution (for concentrations see Table 11)	0.2 µl
ddH_2O	74.98 µl or 68.98 µl (see Table 11)

Deiodination reactions were started by adding 100 µl substrate buffer to each tube and immediately placing the tubes one by one into a water bath at 37 °C. Dio reactions were stopped and processed as described in section 2.3.

2.8.8 Routine quality control samples for Dio assays based on LC-MS/MS

The following control samples were routinely added to each series of samples of LC-MS/MS based Dio assays.

- Positive control samples were Dio reactions containing aliquots of an in-house preparation of mouse liver membrane fractions and 1 µM rT_3 as a well-defined substrate of Dio1 both in the absence and in the presence of 1 mM PTU.
- Negative control samples were Dio reactions containing lysates of Dio activity deficient HEK293 cells and 1 µM rT_3 both in the absence and in the presence of 1 mM PTU.
- Blank samples were Dio reactions containing lysates of heat inactivated cell line lysates or mouse liver membrane fractions depending on the Dio preparation to be used in the main samples and appropriate amounts of DMSO. Blank samples were prepared both in the absence and in the presence of 1 mM PTU.
- Calibration curve samples were prepared as described for blank samples, but instead of DMSO, six appropriate concentrations of the Dio substrate to be investigated and of the putative deiodination products were added. Blank samples were prepared both in the absence and in the presence of 1 mM PTU. Calibration curves were calculated for each analyte as described in sections 2.4.2 and 3.2.5. Thus, specific calibration curves were obtained for each analyte, which also took the analyte specific recovery rates into account.
- Nevertheless, the recoveries of each analyte were monitored in each experiment. For that purpose, calibration curve samples devoid of any substrate were prepared. After the liquid-liquid extraction procedure, analytes were added to the reconstituted residue at the appropriate final concentrations. Recoveries were determined as described in section 2.4.1 with the calibration curve samples serving as set 3 samples and the post-addition samples described here serving as set 2 samples.

2.8.9 Calculation of apparent K_m and v_{max} from Dio assays

In $^{125}I^-$ release assays and LC-MS/MS based Dio assays which were designed to determine apparent K_m and v_{max} values of Dio catalyzed reactions, the following substrates were used at the indicated concentrations:

- 50 nM - 2.0 µM rT_3 for Dio1 from HepG2 lysates
- 200 nM - 4.4 µM rT_3 for Dio1 from mouse liver membrane fractions
- 10 nM - 50 nM rT_3 for Dio2
- 10 nM - 100 nM T_3 for Dio3
- 10 nM - 5 µM rT_3AM, T_3AM, 3-T_1AM and 3-T_1 for the respective Dio isozyme as indicated in Table 19

The specific Dio activities were expressed as mol $^{125}I^-$ released/mg protein/min and mol deiodinated product/mg protein/min for $^{125}I^-$ release assays and LC-MS/MS based Dio assays, respectively. The apparent K_m and v_{max} values were determined by fitting the enzyme kinetic data to the Michaelis-Menten equation by means of nonlinear regression using GraphPad Prism Software version 4.00 (GraphPad, San Diego, California, USA). The values reported for apparent K_m and v_{max} are from three separate experiments performed in triplicate and are presented as mean ± SD. The values obtained for each Dio preparation by either method were compared using Wilcoxon test.

2.9 Functional validation of newly identified TAM substrates

$^{125}I^-$ release assays were used to investigate the effect of TAMs on the 5'-deiodination of rT_3 by Dio1. Mouse liver membrane fractions were incubated for 15 min with 0.2 - 4.4 µM rT_3 and 0.1 - 10.0 µM TAM at each rT_3 concentration used.

The values reported for apparent K_m and v_{max} were determined as described in section 2.8.9 and are presented as mean ± SD from three separate experiments

performed in triplicate. Linear regression lines from Eadie-Hofstee plots are used to display the effects of TAMs on the K_m (represented by slope) and v_{max} (represented by y-intercept) of the 5'-deiodination of rT_3 by Dio1.

K_i values were determined by Dixon plots and linear regression analysis (Fig. 4, right panel). The values reported for K_i are from three separate experiments performed in triplicate and are presented as mean ± SD. The effect of TAMs on the apparent K_m and v_{max} of the conversion of rT_3 by Dio1 from mouse liver membrane fractions was analyzed statistically using Friedman test followed by Dunn's post test. $p < 0.05$ was considered significant.

3 Results

3.1 Detection of TAMs and THs by LC-MS/MS

3.1.1 Optimization of compound-specific mass spectrometric parameters

TAMs and THs were detected using positive electrospray ionization (ESI+) tandem mass spectrometry applying selected reaction monitoring (SRM). The mass spectrum of each analyte was recorded to optimize the compound-specific mass spectrometric working parameters for the detection of the parent ion filtered by the first quadrupole. Likewise, the product ion tandem mass spectrum was recorded to optimize the parameters for the detection of the most intensive product ion filtered by the third quadrupole. Analytes which represented constitutional isomers, such as 3-T_1AM and 3'-T_1AM, were detected using the same mass spectrometric parameters. The optimized parameters are listed in Table 14.

Table 14: List of the optimized compound-specific mass spectrometric working parameters.

compound	(m/z)[a] Q1[b]	(m/z) Q3[c]	DP[d]	FP[e]	EP[f]	CEP[g]	CE[h]	CXP[i]
T_0AM	230.3	213.3	3.0	144.0	5.0	94.9	15.6	5.0
monoiodothyronamines	356.2	339.2	10.0	138.0	6.0	85.0	15.0	8.0
3-T_1AM-d4	360.2	343.2	10.7	169.0	6.0	84.6	15.0	8.1
diiodothyronamines	482.1	465.1	12.0	177.0	7.6	74.9	16.0	9.0
triiodothyronamines	608.0	591.0	19.0	196.0	7.7	64.9	20.1	12.0
T_4AM	733.9	716.9	32.0	235.0	9.4	54.9	25.0	15.0
T_0	274.3	228.3	9.0	122.8	5.6	92.3	24.1	5.0
monoiodothyronines	400.2	354.2	15.0	179.0	6.0	82.6	23.8	9.9
diiodothyronines	526.1	480.1	18.3	186.0	7.5	72.3	25.5	11.3
triiodothyronines	652.0	606.0	26.0	199.0	8.2	63.0	26.7	12.7
T_4	777.9	731.8	33.3	242.0	9.0	49.8	32.0	15.0

[a] (m/z): mass to charge ratio

[b] (m/z) Q1: m/z of parent ion in first quadrupole

[c] (m/z) Q3: m/z of most intensive product ion in third quadrupole

[d] DP: declustering potential (V)

[e] FP: focusing potential (V)

[f] EP: entrance potential (V)

[g] CEP: collision cell entrance potential (V)

[h] CE: collision energy (V)

[i] CXP: collision cell exit potential (V)

Representative spectra including the interpretation of the reported (m/z) values are presented in the supplementary Figure S28. All TAMs showed the transition $(M+H)^+ \rightarrow (M+H-NH_3)^+$ (Figure S28). Thus, this transition was used for the detection of each TAM. By contrast, the transition $(M+H)^+ \rightarrow (M+H-HCOOH)^+$ was used for detection of all THs. All spectra were reproducible as assessed by repeated recording over a time period of 3 months (data not shown).

3.1.2 Chromatographic separation of TAMs and THs

As described in section 3.1.1, analytes which represented constitutional isomers, such as 3-T_1AM and 3'-T_1AM, were detected using the same mass spectrometric parameters. This was in line with studies reporting that the fragmentation of the parent ions of 3-T_1 and 3'-T_1 as well as 3,5-T_2, 3,3'-T_2 and 3',5'-T_2 in the second quadrupole yielded identical product ions, even if the percent contribution of each product ion was unique to each constitutional isomer [Zhang, 06b; Zhang, 06a]. Thus, the distinction of constitutional isomers required their chromatographic separation prior to the detection by MS/MS. Complete chromatographic separation and symmetrical peak shapes of almost all TAMs and THs were achieved using a Synergi Polar-RP 80 Å column (150 mm x 2 mm) and a gradient elution program at a flow rate of 300 µl/min. The Synergi Polar-RP stationary phase was an ether-linked phenyl phase with hydrophilic endcapping. Thus, analyte retention was due to aromatic and reversed phase selectivity. The analytical column was equipped with an Analytical Guard Cartridge System (4.0 mm x 2.0 mm), which served to retain contaminants possibly contained in the mobile phase or in samples. The parameters of the gradient elution program are shown in Figure 6.

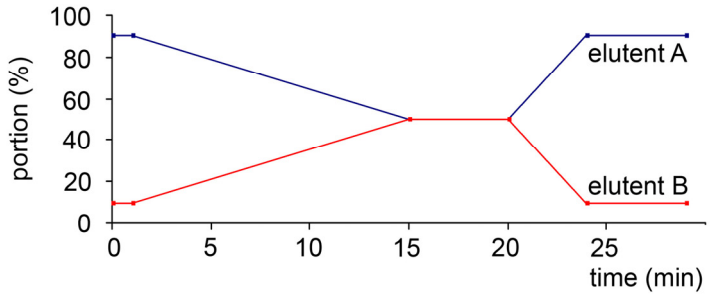
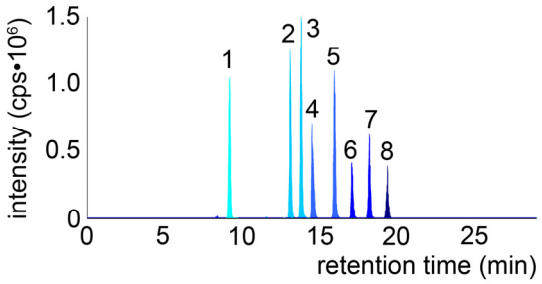


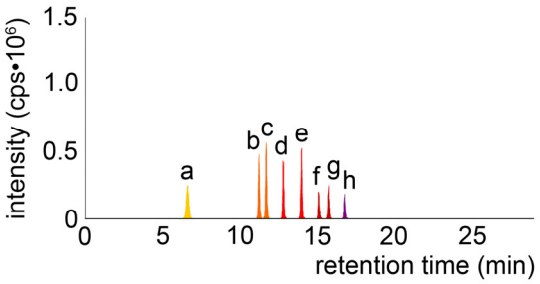
Figure 6: Parameters of the gradient elution program used for the chromatographic separation of TAMs and THs. Eluent A: ddH_2O:acetonitrile:acetic acid (95:5:0.6), eluent B: ddH_2O:acetonitrile: acetic acid (5:95:0.6).

Both 3,3'-T_2AM and 3',5'-T_2AM as well as 3,3'-T_2 and 3',5'-T_2 were separated sufficiently to allow for an unequivocal qualitative identification of each analyte, but baseline peak separation was not achieved by the chromatographic method developed herein. Since neither 3,3'-T_2AM and 3',5'-T_2AM nor 3,3'-T_2 and 3',5'-T_2 were produced simultaneously by any of the Dio catalyzed reactions investigated in this study, the LC-MS/MS method was appropriate to analyze all these reactions both qualitatively and quantitatively. Representative chromatograms of TAM and TH standard solutions including the analyte retention times obtained by the LC-MS/MS method are shown in Figure 7.

A



C



B

index	analyte	retention time (min)
1	T_0AM	9.4
2	3-T_1AM	12.9
3	3'-T_1AM	13.2
4	3,5-T_2AM	14.4
5	3,3'-T_2AM	15.6
6	T_3AM	16.9
7	rT_3AM	17.9
8	T_4AM	19.2

D

index	analyte	retention time (min)
a	T_0	6.9
b	3-T_1	10.9
c	3'-T_1	11.4
d	3,5-T_2	12.5
e	3,3'-T_2	13.8
f	T_3	15.0
g	rT_3	15.5
h	T_4	16.7

E

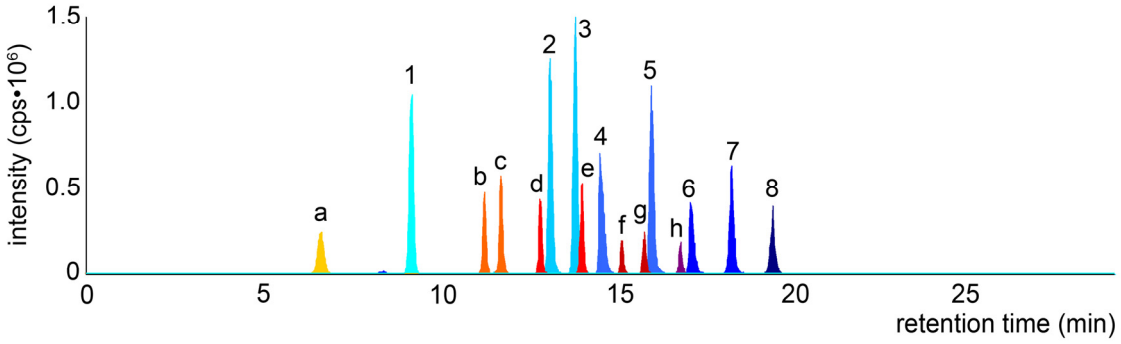


Figure 7: Representative chromatograms and retention times of TAM and TH standard solutions. 30 pmol of T_0AM, monoiodothyronamines (including 3-T_1AM-d4, internal standard, IS), diiodothyronamines, monoiodothyronines and diiodothyronines were injected. Due to the lower intensity of detection of T_0, triiodothyronamines, T_4AM, triiodothyronines and T_4 in this method, 150 pmol of these compounds had to be injected to obtain peaks that were high enough to present them together with the aforementioned analytes in the same chromatogram. 3',5'-T_2AM and 3',5'-T_2 were not injected since their retention times (15.7 min and 13.7 min, respectively) were almost identical to that of 3,3'-T_2AM and 3,3'-T_2, respectively. 30 pmol 3-T_1AM-d4 (IS) were injected, but the respective peak is not visible since its retention time was identical to that of 3-T_1AM. Chromatogram of TAM standard solutions (**A**), identification of TAMs and analyte specific retention times (**B**), chromatogram of TH standard solutions (**C**), identification of THs and analyte specific retention times (**D**), chromatogram of TAM and TH standard solutions (**E**). IS: internal standard.

3.2 Method validation

The LC-MS/MS based analysis of TAMs and THs from biological matrices, such as Dio reactions, required prior extraction of the analytes by a method compatible to LC-MS/MS measurements. A method based on liquid-liquid extraction was developed as described in section 2.3. The detection of extracted analytes by LC-MS/MS was validated according to internationally accepted recommendations [Shah, 00; Peters, 07].

3.2.1 Selectivity

The LC-MS/MS method was found to be selective for all TAM and TH analytes since no interfering peaks were observed in the extracts of blank Dio reactions matrices (Figure 8).

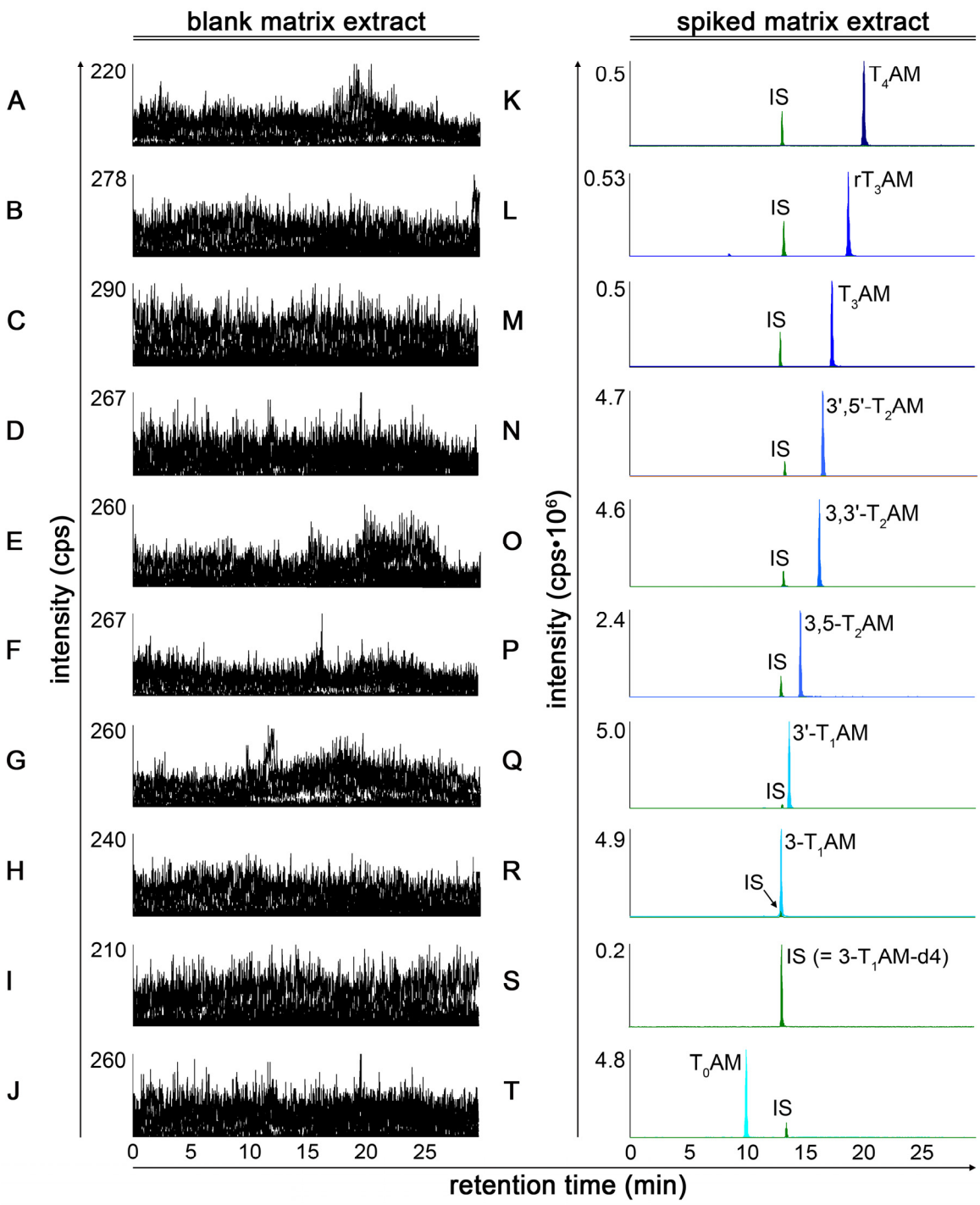


Figure 8: Proof of selectivity of the LC-MS/MS detection of TAM and TH analytes extracted from Dio reactions. Chromatograms of extracts of blank Dio reaction matrices (**left panel**). Exemplary chromatograms of extracts from Dio reactions which contained heat inactivated HepG2 lysates, were devoid of PTU and were spiked with 500 nM individual TAM analytes prior to extraction (**right panel**). IS: internal standard.

3.2.2 Matrix effects

Potential interfering effects of Dio reaction matrices with TAMs and THs were calculated systematically for all Dio reaction matrices and all analytes. Since no relevant matrix effects were observed for any matrix and any analyte (Kruskal-Wallis test), also no differences were detectable between the different Dio reaction matrices (data not shown). Accordingly, the values obtained for Dio reaction matrices which contained HepG2 lysates and were devoid of PTU are shown exemplarily in supplementary Table S20.

3.2.3 Process efficiencies

Since the matrix effects were negligible (section 3.2.2) and the recoveries were independent from analyte concentrations and Dio reaction matrices (section 3.2.4), the values for process efficiencies were also comparable between the different analyte concentrations and matrices. Accordingly, the values obtained for Dio reaction matrices which contained HepG2 lysates and were devoid of PTU are shown exemplarily in supplementary Table S21.

3.2.4 Recoveries

To further validate the performance of the extraction method for TAMs and THs, the recoveries of all analytes were determined. As assessed by Kruskal-Wallis test (data not shown), the recoveries obtained for one analyte did not differ significantly between the various analyte concentrations and the Dio reaction matrices used. Therefore, the recovery data obtained for Dio reactions which contained HepG2 lysates and 500 nM of each analyte but were devoid of PTU are presented exemplarily in Figure 9.

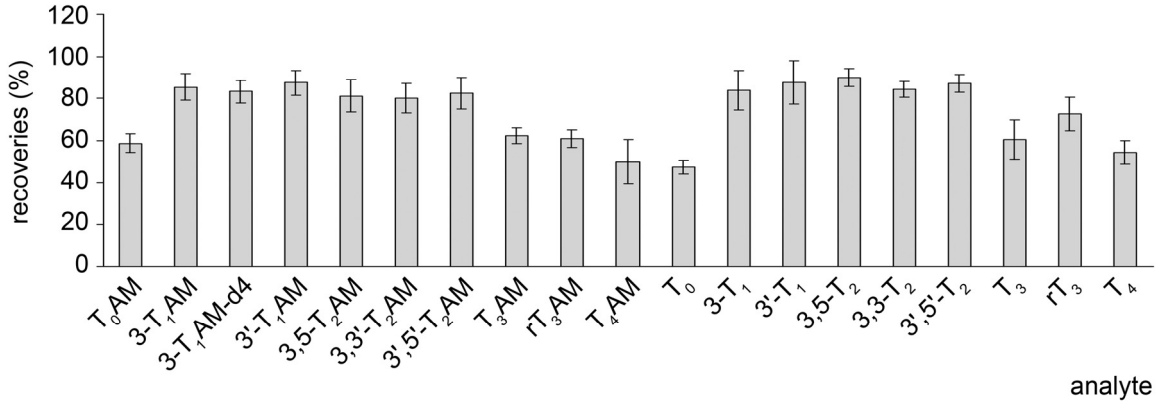


Figure 9: Recoveries of TAM and TH analytes extracted from Dio reaction matrices which contained HepG2 lysates and 500 nM of each analyte but were devoid of PTU.

3.2.5 Linearity

Analyte specific calibration curves were recorded as described in section 2.4.2 in order to determine the linear ranges of the LC-MS/MS method for TAM and TH standard solutions and to validate 3-T_1AM-d4 as an internal standard (IS) for all analytes.

For all analytes and all Dio reaction matrices, homogeneity of variances was achieved using a weighted (1/analyte concentration) least squares model, which was therefore used to calculate the calibration curves. The slopes and y-intercepts of the analyte specific regression lines did not differ between the Dio reaction matrices as determined by Kruskal-Wallis test (data not shown). The coefficients of determination (r^2) were ≥ 0.997 and are listed in Table 15. Thus, the LC-MS/MS method was linear over a wide range of analyte amounts and 3-T_1AM-d4 was verified as an appropriate IS for all TAM and TH analytes.

Table 15: List of analyte specific coefficients of determination (r^2) obtained from calibration curves.

Analyte	r^2	Analyte	r^2
T_0AM	0.9974	T_0	0.991
3-T_1AM	0.9980	3-T_1	0.991
3'-T_1AM	0.9984	3'-T_1	0.995
3-T_1AM-d4	0.9971	3,5-T_2	0.989
3,5-T_2AM	0.9972	3,3'-T_2	0.981
3,3'-T_2AM	0.9972	3',5'-T_2	0.985
3',5'-T_2AM	0.9974	T_3	0.976
T_3AM	0.984	rT_3	0.997
rT_3AM	0.9970	T_4	0.985
T_4AM	0.9978		

Since 3-T_1AM-d4 was validated as a suitable IS for all analytes, the AUCs of TAM and TH analytes will be expressed as follows in all experiments described herein:

$$\text{analyte AUC ratio} = \frac{\text{AUC of analyte}}{\text{AUC of 3-}T_1\text{AM-d4 (IS)}}$$

3.2.6 Limit of detection and limit of quantification

For each analyte, identical LOD and LOQ values were obtained with each Dio reaction matrix. Both parameters were roughly one order of magnitude higher for TAM and TH analytes containing three or four iodine atoms compared to those containing zero to two iodine atoms per molecule with T_0 being an exception as demonstrated in Table 16.

Table 16: List of LOD and LOQ values of TAM and TH analytes extracted from Dio reaction matrices.

Analyte	LD	LOQ	Analyte	LD	LOQ
T_0AM	100 pM	250 pM	T_0	1 nM	2.5 nM
3-T_1AM	100 pM	250 pM	3-T_1	250 pM	500 pM
3-T_1AM-d4	100 pM	250 pM	3'-T_1	250 pM	500 pM
3'-T_1AM	100 pM	250 pM	3,5-T_2	250 pM	500 pM
3,5-T_2AM	250 pM	500 pM	3,3'-T_2	250 pM	500 pM
3,3'-T_2AM	100 pM	250 pM	3',5'-T_2	250 pM	500 pM
3',5'-T_2AM	100 pM	250 pM	T_3	2.5 nM	5 nM
T_3AM	1 nM	2.5 nM	rT_3	2.5 nM	5 nM
rT_3AM	1 nM	2.5 nM	T_4	2.5 nM	5 nM
T_4AM	1 nM	2.5 nM			

3.2.7 Precision and bias

Several stability parameters were determined to verify that the LC-MS/MS method worked reproducibly both on a within-day as well as day-to-day basis as described in (sections 2.4.4 and 2.4.5). Both *intra*- and *inter-assay* precision and bias did not differ significantly between the different concentrations of each analyte and the Dio reaction matrices as determined by Kruskal-Wallis test (data not shown). Accordingly, the analyte specific data obtained for PTU-free Dio reactions containing HepG2 lysates and 500 nM of each analyte are presented in Table 17. *Intra*- and *inter-assay* precision of retention times were < 2.14 sec and < 2.18 sec, respectively. *Intra*- and *inter-assay* precision of analyte concentrations ranged between 5.0 to 8.3 % and 5.6 to 11.0 %, respectively. Thus, the precision data were within the required limits of 15 % coefficient of variation, at all analyte concentrations studied [Shah, 00; Peters, 07]. *Intra*- and *inter-assay* bias of analyte concentrations ranged between 3.3 to 10.3 % and 6.1 to 12.4 %. Thus, bias data were also within the acceptance range of ± 15 % of the nominal values at all concentrations [Shah, 00; Peters, 07].

Table 17: List of precision of analyte retention times as well as precision and bias of analyte concentrations as obtained for both *intra-* and *inter-assay* conditions.

Analyte	***Intra-assay* variation**			***Inter-assay* variation**		
	Precision of rt (s) [a]	Precision of [analyte] (%)	Bias of [analyte] (%)	Precision of rt (s) [a]	Precision of [analyte] (%)	Bias of [analyte] (%)
T_0AM	2.14	8.3	5.2	2.18	11.0	6.1
3-T_1AM	1.64	5.9	3.9	1.36	6.6	6.9
3'-T_1AM	0.99	4.2	4.6	1.64	4.5	7.2
3,5-T_2AM	1.50	5.1	3.3	1.49	9.3	8.1
3,3'-T_2AM	1.34	5.0	3.2	1.73	5.8	8.5
3',5'-T_2AM	1.46	5.3	3.9	1.46	5.4	6.4
T_3AM	1.57	5.0	7.8	1.42	5.6	13.7
rT_3AM	1.36	4.6	5.9	2.09	4.9	7.2
T_4AM	1.33	7.1	10.3	1.60	9.1	11.9
T_0	2.01	7.3	8.8	2.12	10.8	10.2
3-T_1	1.38	5.1	6.4	1.27	5.4	7.4
3'-T_1	1.79	5.2	7.0	1.64	5.7	4.6
3,5-T_2	1.25	5.5	2.4	1.35	6.4	6.3
3,3'-T_2	1.55	5.5	3.8	1.36	6.3	6.9
3',5'-T_2	1.32	4.5	5.1	1.24	5.1	8.9
T_3	1.36	6.3	8.1	1.74	10.5	12.7
rT_3	1.46	5.9	9.4	1.78	10.0	6.3
T_4	1.83	5.2	3.9	1.28	10.7	12.4

[a] The precision of the retention time is presented as standard deviation.

[b] By contrast, the precision of the analyte concentration is presented as coefficient of variation.

rt = retention time

3.2.8 Analyte stability in extracted Dio assays

The stability of TAM and TH analytes in extracted Dio assays was assessed as described in section 2.4.6 and did not differ between the analyte concentration levels and the Dio reaction matrices (Kruskal-Wallis test). Since the analyte spe-

cific coefficients of variation never exceeded the *inter-assay* precisions of analyte concentration (data not shown), the analytes were considered as stable under the autosampler and storage conditions used in this study. Thus, non-enzymatic deiodinations under autosampler and storage conditions were excluded.

3.2.9 Long-term stability of TAM and TH stock solutions

The long-term stability of TAM and TH stock solutions, which were prepared as described in section 2.2.2, was monitored over a period of 2 years as depicted in section 2.4.7. Since the analyte specific coefficients of variation never exceeded the *intra-assay* precisions of analyte concentration (data not shown), the TAM and TH stock solutions were considered as stable.

3.3 Isozyme specificity of Dio preparations

The following human cell lines and tissues were selected as well-established sources for endogenous high specific activities of the respective Dio isozyme:

- HepG2 for Dio1
- mouse liver for Dio1
- MSTO-211H for Dio2
- ECC-1 for Dio3

Still, to verify the exclusive expression of the respective Dio isozyme in each preparation, the following experiments were performed:

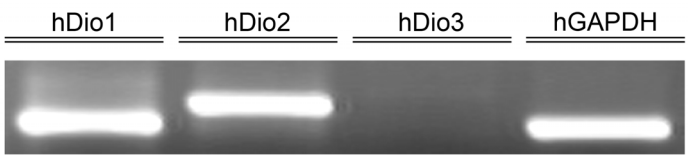
- Dio transcripts were analyzed using semiquantitative RT-PCR.
- Apparent K_m and v_{max} of Dio1 and Dio2 activities were measured using $^{125}I^-$ release assays employing rT_3 as substrate. Additionally, apparent K_m and v_{max} of Dio1 and Dio2 activities were measured using the novel liquid-liquid extraction and LC-MS/MS detection protocol also employing rT_3 as substrate. The

values obtained by both methods were compared using Wilcoxon test thus validating the performance of the novel LC-MS/MS method in Dio assays.

- Apparent K_m and v_{max} of Dio3 activities were measured using LC-MS/MS based Dio assays and employing T_3 as substrate. The specific activities obtained for Dio3 in ECC-1 lysates were compared to the values obtained by a radiochemical, HPLC-based assay [Kester, 06].
- The substrate specificity of the Dio preparations towards all iodothyronines, namely T_4, rT_3, T_3, 3',5'-T_2, 3,3'-T_2, 3,5-T_2, 3'-T_1 and 3-T_1, was analyzed using LC-MS/MS based Dio assays.

3.3.1 HepG2 lysates as a source for Dio1

In HepG2 lysates, Dio1 transcript and enzymatic activity were detected (Figure 10). The apparent K_m and v_{max} values of the specific Dio1 activity with rT_3 as obtained by $^{125}I^-$ release assay and by LC-MS/MS assay were typical of Dio1 [Köhrle, 02] and did not differ significantly (Wilcoxon test; Figure 10B, C). Although Dio2 transcript was detected, no corresponding PTU-insensitive specific Dio2 activity was measured. Such discrepancies between specific activity and cognate transcript are not unusual for selenoproteins, such as Dio isozymes, since successful selenoprotein translation is very complex, error-prone and less efficient than that of typical proteins [Gereben, 02; Dumitrescu, 05]. Dio3 expression was neither detected at the transcript nor at the activity level (Figure 10), which was in line with published data [Kester, 06].



A	Dio transcripts				
B	Dio activities ($^{125}I^-$ release)				
	K_m (µM rT_3 or T_3)	0.59 ± 0.03	nd	nd	na
	v_{max} (pmol I^-/mg/min)	2.2 ± 0.06	nd	nd	na
C	Dio activities (LC-MS/MS)				
	K_m (µM rT_3 or T_3)	0.57 ± 0.05	nd	nd	na
	v_{max} (pmol 3,3'-T_2/mg/min)	2.5 ± 0.11	nd	nd	na

Figure 10: Expression of Dio transcripts in HepG2 cells as determined by RT-PCR (**A**) and quantification of specific Dio enzymatic activities in HepG2 lysates as determined by $^{125}I^-$ release assays (**B**) or LC-MS/MS based Dio assays (**C**). nd: not detected, na: not applicable.

Incubation of HepG2 lysates with iodothyronine substrates revealed that T_4 was weakly 5'-deiodinated to T_3 (Figure 11A), which is consistent with previous findings [Chopra, 76; Visser, 78; Kaplan, 78]. Furthermore, a small 3,3'-T_2 peak was observed. Theoretically, 3,3'-T_2 might have been formed by further 5-deiodination of T_3 or by 5-deiodination of T_4 to rT_3 and subsequent rapid 5'-deiodination of rT_3. The latter interpretation is consistent with published data [Visser, 79; Moreno, 94] and supported by the finding that rT_3 was almost completely 5'-deiodinated to 3,3'-T_2 by Dio1 preparations from HepG2 cells whereas T_3 was not deiodinated at all (Figure 11B, C). Apart from rT_3, 3',5'-T_2 was also 5'-deiodinated to 3'-T_1 (Figure 11D). The deiodinations of T_4, rT_3 and 3',5'-T_2 were completely inhibited by the Dio1 specific inhibitor PTU, which is in line with published Dio1 inhibitions characteristics [Köhrle, 02]. Both 3-T_1 and 3'-T_1 were no substrates of Dio1 from HepG2 lysates, which is consistent with previously reported data [Chopra, 81; Otten, 84; Sorimachi, 80a].

However, some TH deiodination reactions which have been described for Dio1, namely the 3'-deiodination of 3,3'-T_2 as well as the 5-deiodinations of T_3 and 3,5-T_2 [Kuiper, 05; Chopra, 82], were not catalyzed by Dio1 from HepG2 lysates. Therefore, HepG2 lysates were used as an isozyme specific source of Dio1 in

further experiments, but an additional Dio1 preparation, namely mouse liver membrane fractions, was added to the analyses.

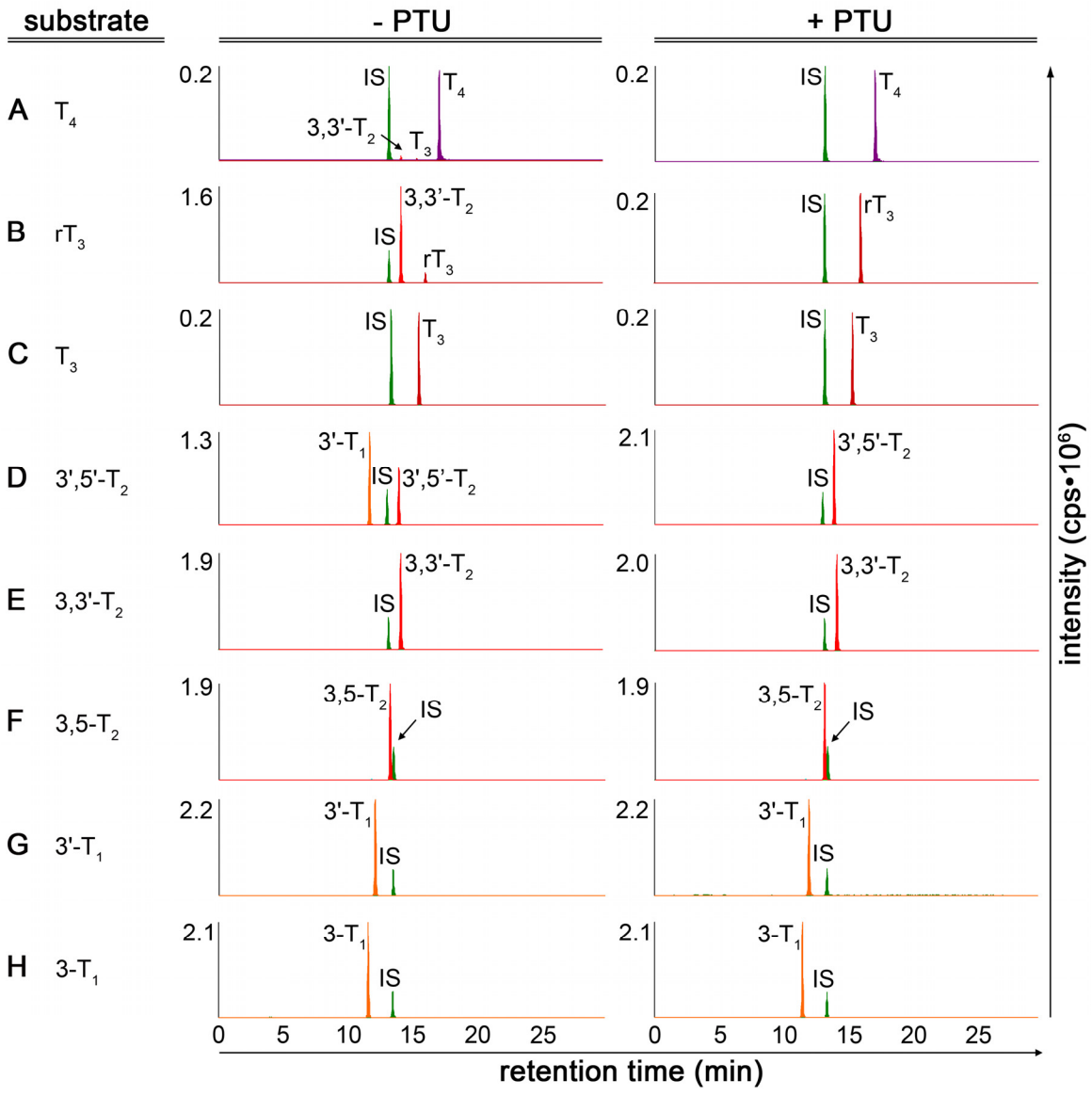


Figure 11: Chromatograms of LC-MS/MS based Dio assays using HepG2 lysates as Dio1 specific preparations and 500 nM of the indicated iodothyronine as substrate. IS: internal standard.

The analyte AUC ratio in PTU containing HepG2 incubations never differed from that measured in negative control samples, which contained either heat inacti-

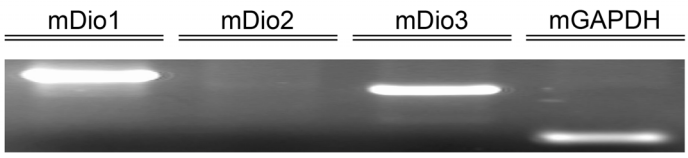
vated HepG2 lysates or Dio deficient HEK293 lysates (see section 2.8.8, data not shown). This was also observed for TAM substrates and for mouse liver membrane fractions. Likewise, the TAM and TH analyte AUC ratios in heat inactivated MSTO-211H and ECC-1 lysates were never different from that measured in HEK293 lysates. Accordingly, with the reaction conditions used herein, the TAM and TH substrates were only subject to deiodination reactions but not to alternate non-deiodinative pathways reported for THs, such as sulfation, glucuronidation, ether-link cleavage or side chain modifications, such as decarboxylation and oxidative deamination [Wu, 05].

Furthermore, with each deiodination reaction analyzed herein, the amount of product(s) formed was always, without any exception, identical to the amount of substrate converted (data not shown).

3.3.2 Mouse liver membrane fractions as a source for Dio1

Liver membrane fractions from male C57BL/6 mice were used as a second source of Dio1 enzyme activity in this study since Dio1 from HepG2 cells did not catalyze all TH deiodination reactions which have been reported for Dio1 (section 3.3.1). In mouse liver membrane fractions, Dio1 transcript and specific activity were detected (Figure 12A, B), which was consistent with previous data [Schneider, 06]. Again, $^{125}I^-$ release assays and LC-MS/MS assays employing rT_3 as substrate yielded apparent K_m values in the higher nanomolar range and high apparent v_{max} values (Figure 12B, C), which were typical of Dio1 activity [Schoenmakers, 92] and did not differ significantly between the two assays used (Wilcoxon test). Dio2 expression was neither detected at the transcript nor at the enzymatic activity level (Figure 12), which was in line with Dio2 expression reported for rodent liver [Schneider, 06; Bates, 99]. By contrast, a Dio3 transcript was detected in mouse liver, while no corresponding specific Dio3 activity was measured (Figure 12). These findings were consistent with the detection of a Dio3 transcript in the liver of 6 to 12 week old female C57BL/6 mice but inconsis-

tent with the measurement of cognate specific Dio3 activity [Boelen, 05]. However, the specific activity reported in the cited study was very low (0.5 fmol/mg protein/min at a substrate concentration of 1 nM T_3). Besides, it was in disagreement with several studies on rat Dio3 expression, which have reported that specific Dio3 activity was highest in the fetus, decreased rapidly after birth and was absent in the adult [Bates, 99; Galton, 91; Huang, 88].



A	Dio transcripts				
B	Dio activities ($^{125}I^-$ release)				
	K_m (µM rT_3 or T_3)	0.43 ± 0.02	nd	nd	na
	v_{max} (pmol I^-/mg/min)	142.7 ± 2.7	nd	nd	na
C	Dio activities (LC-MS/MS)				
	K_m (µM rT_3 or T_3)	0.46 ± 0.09	nd	nd	na
	v_{max} (pmol 3,3'-T_2/mg/min)	147.1 ± 4.3	nd	nd	na

Figure 12: Expression of Dio transcripts in mouse liver as determined by RT-PCR (**A**) and quantification of specific Dio enzymatic activities in mouse liver membrane fractions as determined by $^{125}I^-$ release assays (**B**) or LC-MS/MS based Dio assays (**C**). nd: not detected, na: not applicable.

As observed with Dio1 from HepG2 lysates, mouse liver membrane fractions also deiodinated T_4 yielding T_3 and 3,3'-T_2 (Figure 13A). Likewise, rT_3 and 3',5'-T_2 were 5'-deiodinated (Figure 13B, D). In addition to Dio1 from HepG2 lysates, a weak 3'-deiodination of 3,3'-T_2 was observed as well as faint 5-deiodinations of both T_3 and 3,5-T_2 (Figure 13C, E, F). All reactions were inhibited by PTU. The monoiodothyronines were the only THs not to be accepted as substrates of Dio1 in these studies. Accordingly, no substrate disappearance was monitored in these reactions (Figure 13G, H).

In summary, mouse liver membrane fractions catalyzed all TH deiodination reactions which have been described for Dio1, so far (compare Figure 4 to Figure 13).

Therefore, they were employed as an additional Dio1 specific preparation in further experiments.

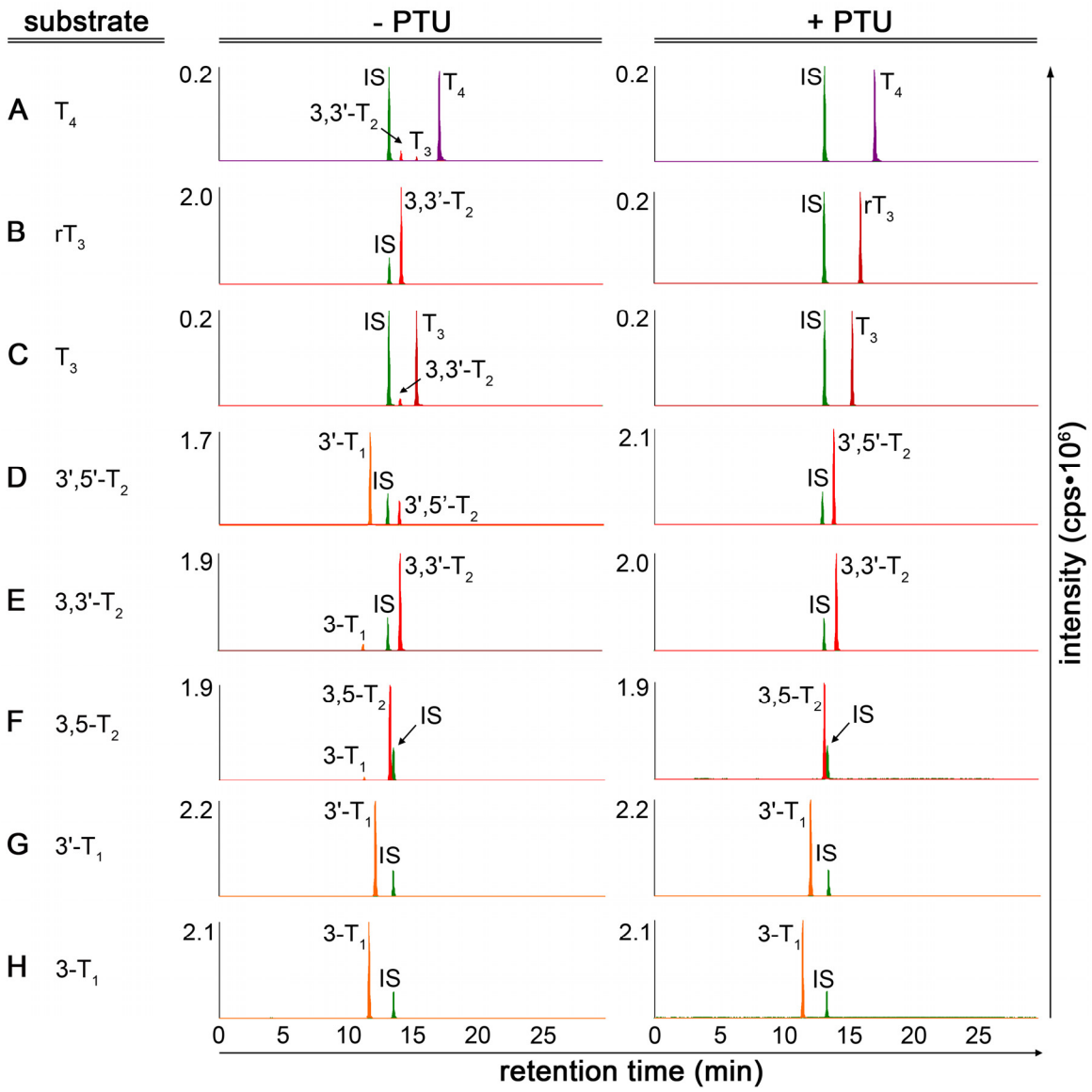
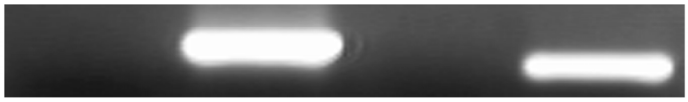


Figure 13: Chromatograms of LC-MS/MS based Dio assays using mouse liver membrane fractions as Dio1 specific preparations and 500 nM of the indicated iodothyronine as substrate. IS: internal standard.

3.3.3 MSTO-211H lysates as a source for Dio2

MSTO-211H cells expressed the Dio2 transcript exclusively (Figure 14A). Furthermore, the typical nanomolar K_m reported for Dio2 [Köhrle, 02] and the high v_{max} previously reported for MSTO-211H cells were measured using $^{125}I^-$ release assays and LC-MS/MS based Dio assays with rT_3 as substrate [Curcio, 01] (Figure 15B, C).



A	Dio transcripts				
B	Dio activities ($^{125}I^-$ release)				
	K_m (µM rT_3 or T_3)	nd	0.005 ± 0.001	nd	na
	v_{max} (pmol I^-/mg/min)	nd	0.130 ± 0.026	nd	na
C	Dio activities (LC-MS/MS)				
	K_m (µM rT_3 or T_3)	nd	0.003 ± 0.002	nd	na
	v_{max} (pmol 3,3'-T_2/mg/min)	nd	0.124 ± 0.031	nd	na

Figure 14: Expression of Dio transcripts in MSTO-211H cells as determined by RT-PCR (**A**) and quantification of specific Dio enzymatic activities in MSTO-211H lysates as determined by $^{125}I^-$ release assays (**B**) or LC-MS/MS based Dio assays (**C**). nd: not detected, na: not applicable.

T_4, rT_3 and 3',5'-T_2 were 5'-deiodinated by MSTO-211H lysates in a PTU-insensitive fashion (Figure 15A, B, D), which is in line with known inhibition characteristics of Dio2 [Köhrle, 02] (section 1.2.2). T_3, 3,3'-T_2 and 3'-T_1, which have either not been reported or been excluded as Dio2 substrates, so far, were also not deiodinated in these experiments (Figure 15C, E, G). Since Dio2 catalyzes phenolic ring deiodinations exclusively, iodothyronines containing only tyrosyl ring iodine atoms, namely 3,5-T_2 and 3-T_1, served as negative control substrates. Indeed, neither substrate disappearance nor product formation were observed in incubations with these two compounds (Figure 15F, H). Taken together, the substrate specificity of MSTO-211H lysates in these assays mirrored that reported for

Dio2. Therefore, MSTO-211H lysates represented an isozyme specific preparation of Dio2.

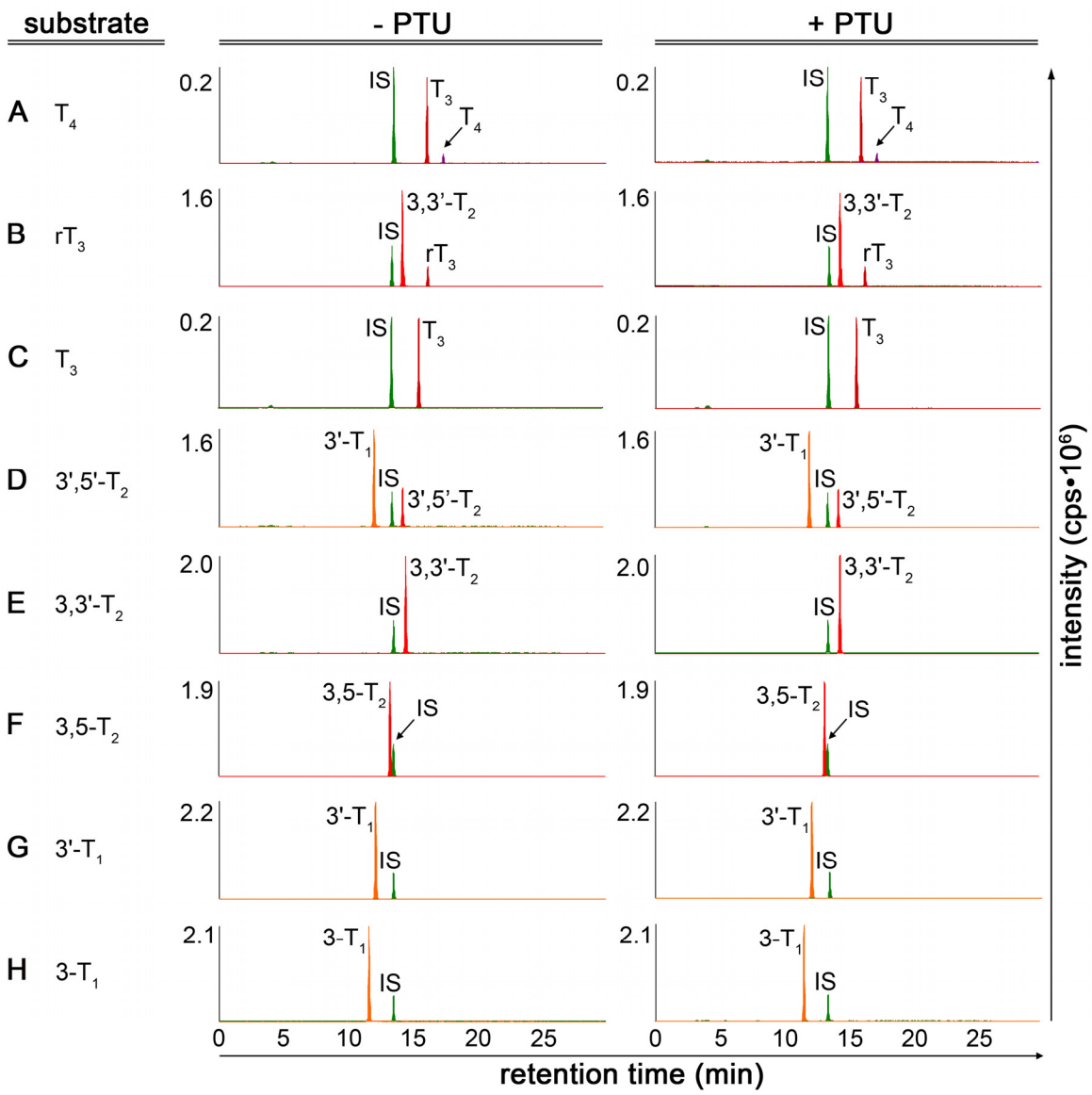
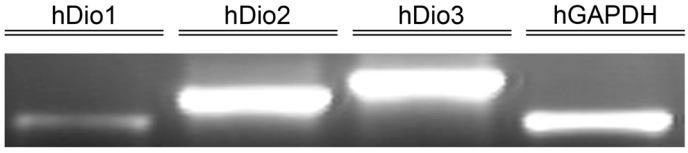


Figure 15: Chromatograms of LC-MS/MS based Dio assays using MSTO-211H lysates as Dio2 specific preparations and 500 nM of the indicated iodothyronine as substrate. IS: internal standard.

3.3.4 ECC-1 lysates as a source for Dio3

In ECC-1 lysates, both Dio3 transcript and enzymatic activity were detected (Figure 16A, C). The specific Dio3 activities measured in LC-MS/MS based Dio assays were in line with those obtained by a radiochemical, HPLC-based assay [Kester, 06]. Just like in HepG2 cells, no Dio2 enzymatic activity was measured in ECC-1 cells despite the detection of a Dio2 transcript. Since Dio1 mRNA and minimal Dio1 enzymatic activity were found in ECC-1 lysates (specific Dio1 activity of 23 ± 3 fmol $^{125}I^-$ released/mg protein/min at a final rT_3 concentration of 1.0 µM), they were used as a specific source for Dio3 only in the presence of 1 mM PTU as an efficient inhibitor of Dio1.



A	Dio transcripts				
B	Dio activities ($^{125}I^-$ release)				
	K_m (µM rT_3 or T_3)	see text	nd	na	na
	v_{max} (pmol I^-/mg/min)	see text	nd	na	na
C	Dio activities (LC-MS/MS)				
	K_m (µM rT_3 or T_3)	see text	nd	0.006 ± 0.002	na
	v_{max} (pmol 3,3'-T_2/mg/min)	see text	nd	2.9 ± 0.2	na

Figure 16: Expression of Dio transcripts in ECC-1 cells as determined by RT-PCR (**A**) and quantification of specific Dio enzymatic activities in ECC-1 lysates as determined by $^{125}I^-$ release assays (**B**) or LC-MS/MS based Dio assays (**C**). nd: not detected, na: not applicable.

The isozyme specificity of such ECC-1 lysates containing 1 mM PTU was verified by assessing their substrate specificity towards the complete panel of iodothyronines. Tyrosyl ring deiodinations of all iodothyronines containing tyrosyl ring iodine atoms, namely T_4, rT_3, T_3, 3,3'-T_2, 3,5-T_2 and 3-T_1, were observed (Figure 17A, B, C, E, F, H). Since these reactions were not inhibited by PTU, they were unambiguously catalyzed by Dio3. Apart from 3-T_1, which has not been reported as a Dio3 substrate yet, all of the observed tyrosyl ring deiodinations have already

been reported as Dio3 catalyzed reactions [Köhrle, 02; Sorimachi, 79a; Sorimachi, 79b; Sorimachi, 77]. Iodothyronines containing only phenolic ring iodine atoms, namely 3',5'-T_2 and 3'-T_1, served as negative control substrates since Dio3 catalyzes tyrosyl ring deiodinations exclusively. Indeed, neither substrate disappearance nor product formation was observed in incubations with these two compounds (Figure 17D, G). Taken together, the reactions catalyzed by Dio3 from ECC-1 lysates were in line with the substrate specificity reported for Dio3 towards THs. Based on these experiments, ECC-1 lysates containing 1 mM PTU were considered as an isozyme specific preparation of Dio3.

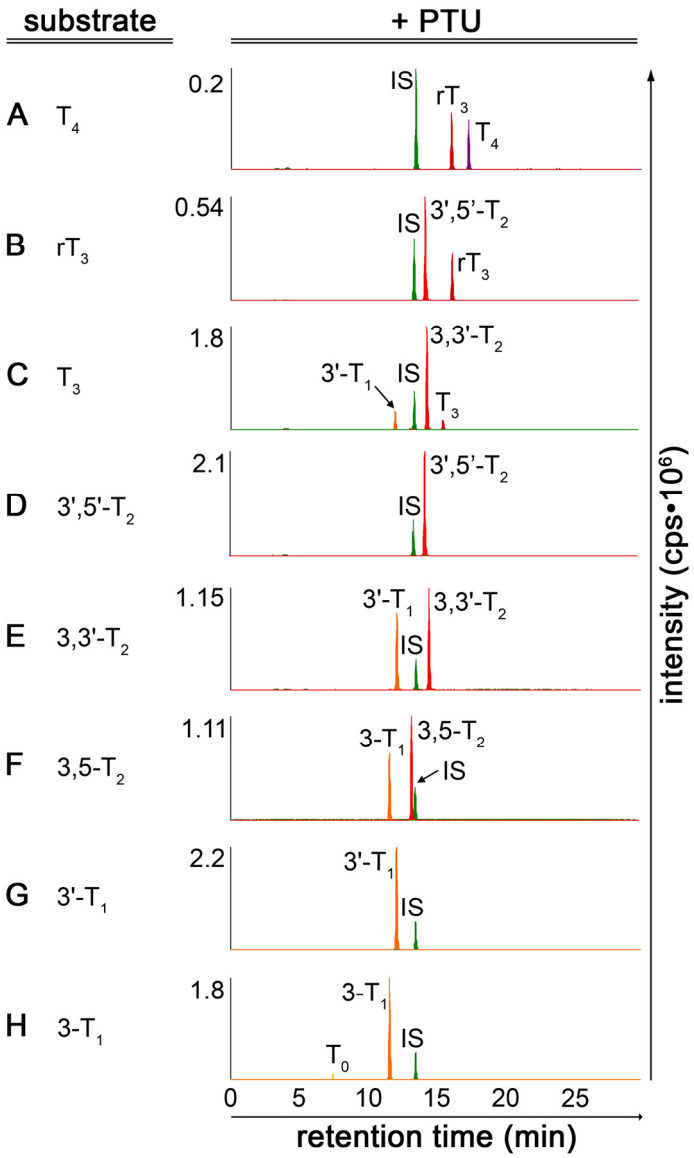
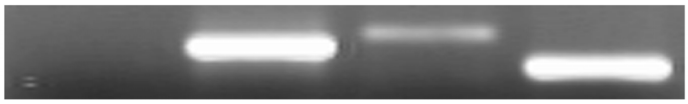


Figure 17: Chromatograms of LC-MS/MS based Dio assays using ECC-1 lysates as Dio3 specific preparations and 500 nM of the indicated iodothyronine as substrate. Since minimal Dio1 enzymatic activity was found in ECC-1 lysates, they were used as a specific source for Dio3 only in the presence of 1 mM PTU as an efficient inhibitor of Dio1. IS: internal standard.

3.3.5 HEK293 lysates as negative controls

HEK293 lysates served as negative control preparations since no Dio activities were measured despite a strong signal for Dio2 mRNA and a faint band for Dio3

transcript (Figure 18). Moreover, none of the iodothyronine substrates was converted by HEK293 lysates (data not shown).



A	Dio transcripts				
B	Dio activities ($^{125}I^-$ release)				
	K_m (µM rT_3 or T_3)	nd	nd	nd	na
	v_{max} (pmol I^-/mg/min)	nd	nd	nd	na
C	Dio activities (LC-MS/MS)				
	K_m (µM rT_3 or T_3)	nd	nd	nd	na
	v_{max} (pmol 3,3'-T_2/mg/min)	nd	nd	nd	na

Figure 18: Expression of Dio transcripts in HEK293 cells as determined by RT-PCR (**A**) and quantification of specific Dio enzymatic activities in HEK293 lysates as determined by $^{125}I^-$ release assays (**B**) or LC-MS/MS based Dio assays (**C**). nd: not detected, na: not applicable.

3.4 Identification of TAM deiodination reactions

3.4.1 TAM substrates of Dio1 containing HepG2 lysates

To test the ability of Dio1 from HepG2 cells to accept TAMs as substrates, HepG2 lysates were incubated as described in Table 11 using various concentrations of TAMs as substrates. Both rT_3AM and 3',5'-T_2AM were readily 5'-deiodinated to 3,3'-T_2AM and 3'-T_1AM, respectively at all substrate concentrations tested (Figure 19B, D). As both reactions were sensitive to PTU inhibition, they were unambiguously catalyzed by Dio1. No conversions of rT_3AM and 3',5'-T_2AM were observed in negative control experiments performed with HEK293 lysates or heat-inactivated HepG2 lysates (data not shown). All other iodothyronamines were not deiodinated by HepG2 cell lysates under these conditions and at the substrate concentrations tested (Figure 19A, C, E -H). The finding that T_4AM was no substrate of Dio1 was in line with a previous study [Köhrle, 84].

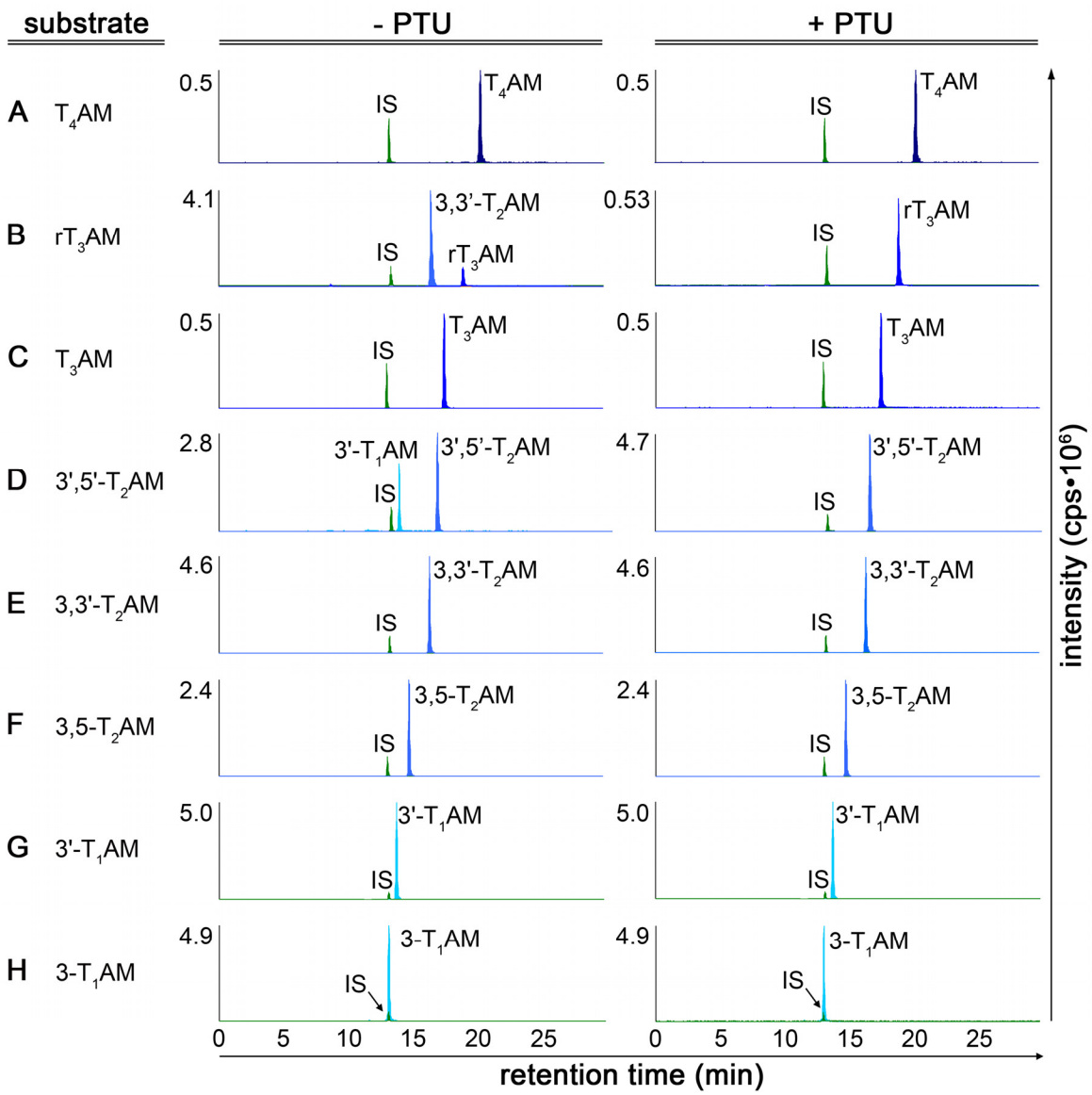


Figure 19: Chromatograms of LC-MS/MS based Dio assays using HepG2 lysates as Dio1 specific preparations and 500 nM of the indicated iodothyronamine as substrate. IS: internal standard.

3.4.2 TAM substrates of Dio1 containing mouse liver membrane fractions

In line with HepG2 experiments, rT_3AM and 3',5'-T_2AM were 5'-deiodinated in a PTU-sensitive way (Figure 20A, B). In contrast to the HepG2 assays, 3,3'-T_2AM was 3'-deiodinated yielding 3-T_1AM (Figure 20C). In addition, weak 5-

deiodinations at the tyrosyl rings of T_3AM and 3,5-T_2AM gave rise to 3,3'-T_2AM and 3-T_1AM, respectively (Figure 20D, E).

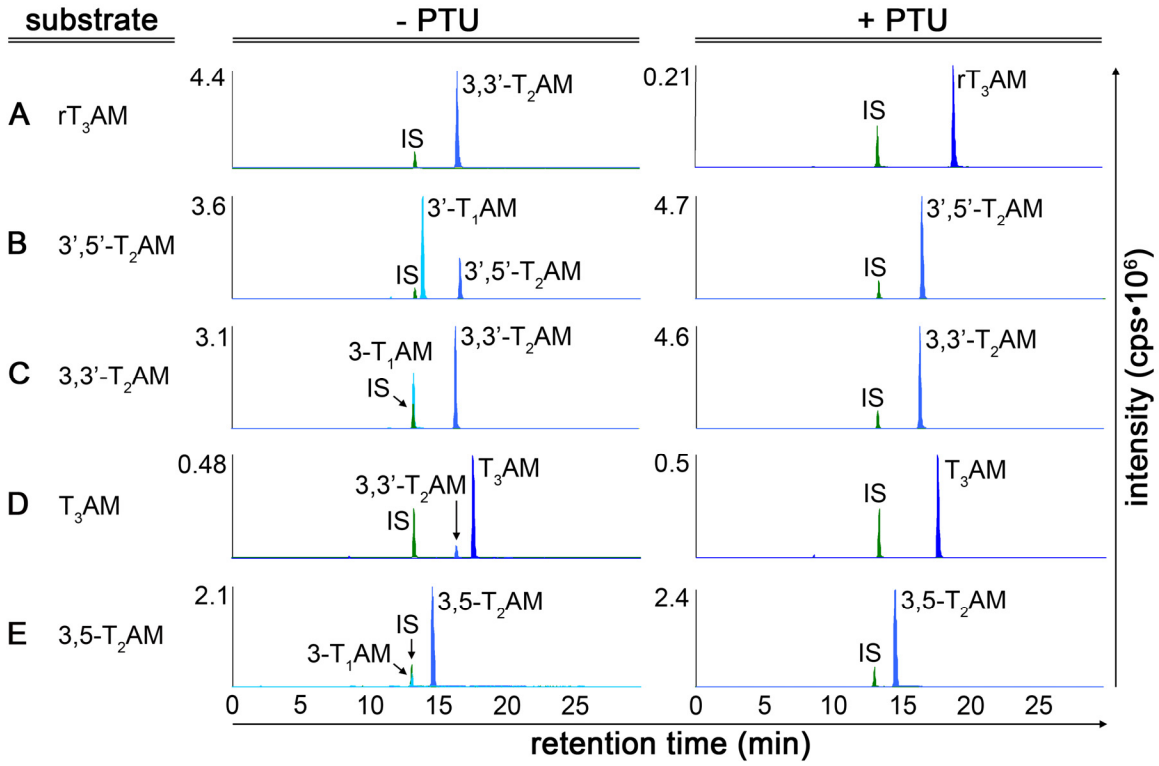


Figure 20: Chromatograms of LC-MS/MS based Dio assays using mouse liver membrane fractions as Dio1 specific preparations and 500 nM of the indicated iodothyronamine as substrate. IS: internal standard.

3.4.3 TAM substrates of Dio2 containing MSTO-211H lysates

Of all iodothyronamines, only rT_3AM and 3',5'-T_2AM were 5'-deiodinated by Dio2 from MSTO-211H lysates yielding 3,3'-T_2AM and 3'-T_1AM, respectively (Figure 21). In contrast to HepG2 lysates, these 5'-deiodination reactions were not sensitive to PTU, which is in agreement with the known inhibition characteristics of Dio2.

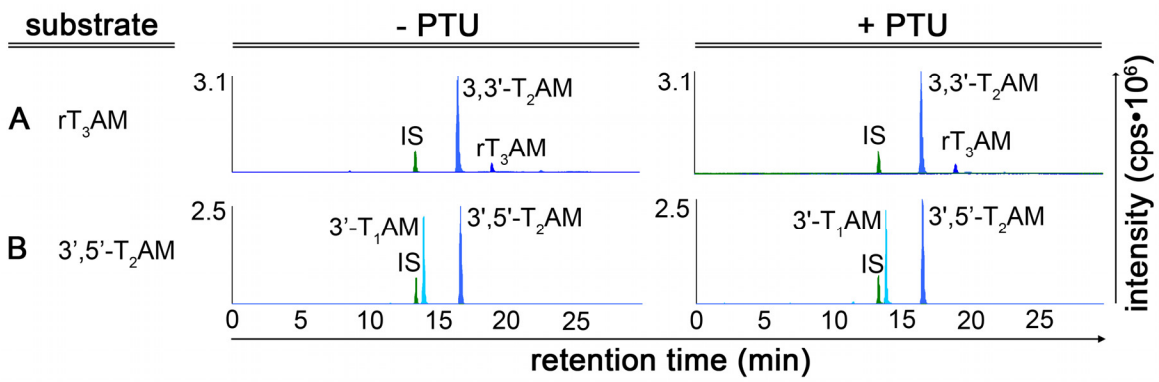


Figure 21: Chromatograms of LC-MS/MS based Dio assays using MSTO-211H lysates as Dio2 specific preparations and 500 nM of the indicated iodothyronamine as substrate. IS: internal standard.

3.4.4 TAM substrates of Dio3 containing ECC-1 lysates

To inhibit the weak Dio1 enzymatic activity that had been found in ECC-1 cells, 1 mM PTU was used throughout the incubations. Tyrosyl ring deiodinations of all TAMs (Figure 22) except those without tyrosyl ring iodine, namely 3',5'-T_2AM and 3'-T_1AM, were observed (data not shown). As these reactions occurred in the presence of PTU, they were clearly catalyzed by Dio3.

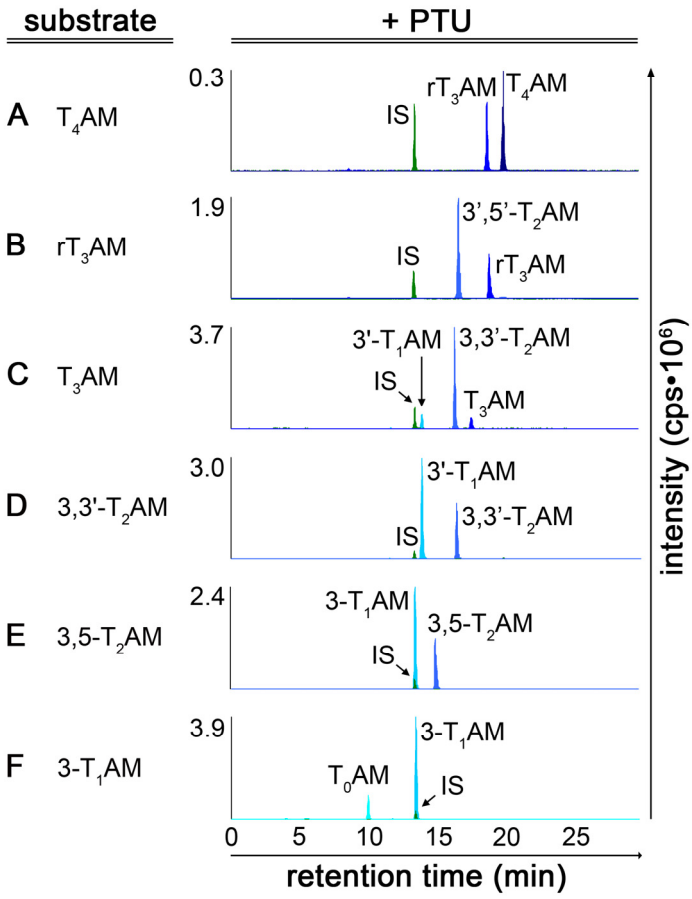


Figure 22: Chromatograms of LC-MS/MS based Dio assays using ECC-1 lysates as Dio3 specific preparations and 500 nM of the indicated iodothyronamine as substrate. Since minimal Dio1 enzymatic activity was found in ECC-1 lysates they were used as a specific source for Dio3 only in the presence of 1 mM PTU as an efficient inhibitor of Dio1. IS: internal standard.

3.4.5 Summary of Dio substrate specificities towards TAMs

The newly identified TAM deiodination reactions catalyzed by each Dio isozyme are summarized in Figure 23.

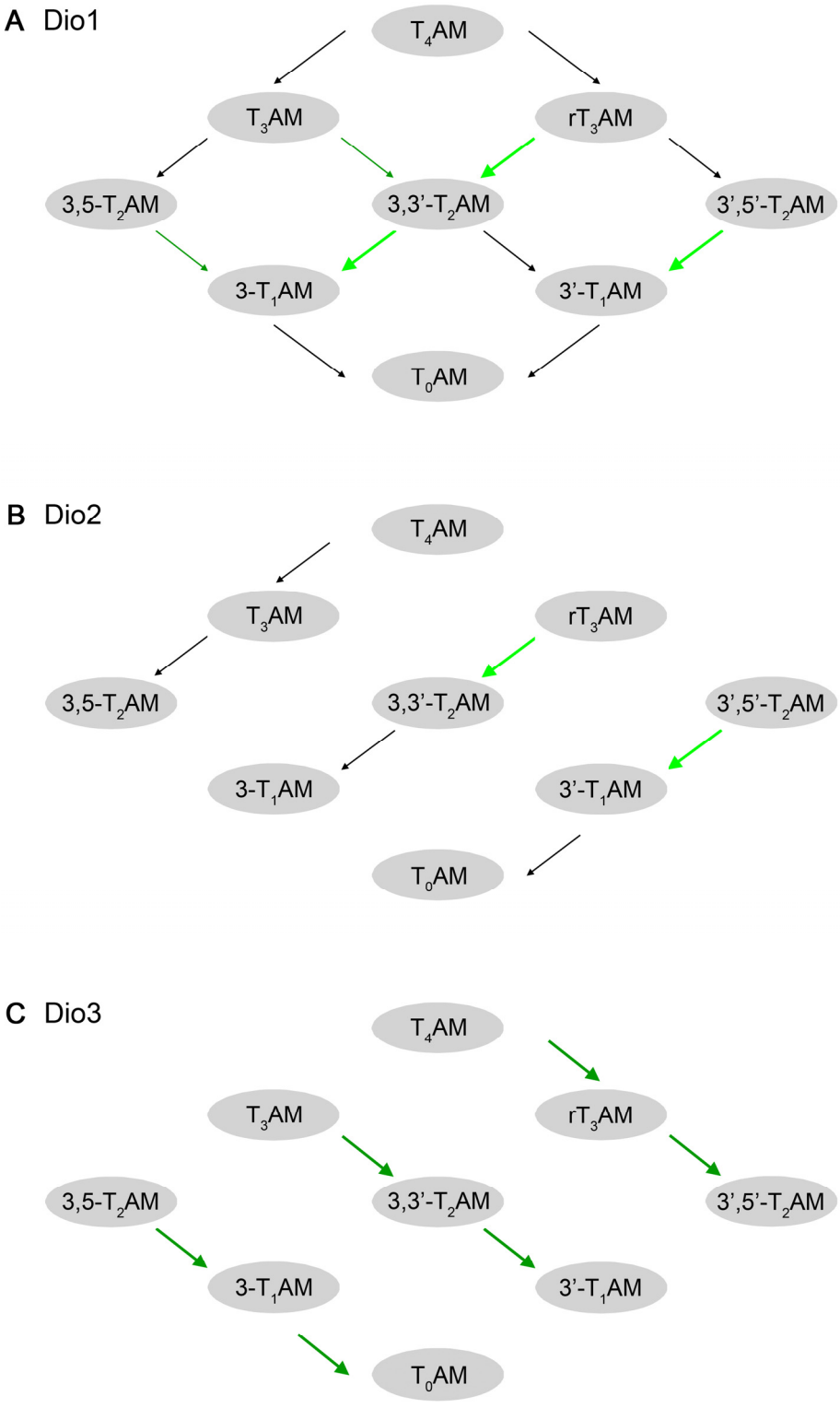

A Dio1
T4AM
T3AM
rT3AM
3,5-T2AM
3,3'-T2AM
3',5'-T2AM
3-T1AM
3'-T1AM
T0AM
B Dio2
T4AM
T3AM
rT3AM
3,5-T2AM
3,3'-T2AM
3',5'-T2AM
3-T1AM
3'-T1AM
T0AM
C Dio3
T4AM
T3AM
rT3AM
3,5-T2AM
3,3'-T2AM
3',5'-T2AM
3-T1AM
3'-T1AM
T0AM

Figure 23: Summary of the newly identified TAM deiodination reactions catalyzed by each Dio isozyme. Arrows: pointing to lower left: phenolic ring deiodinations, pointing to lower right: tyrosyl ring deiodinations, light green: phenolic ring deiodinations catalyzed by the respective Dio isozyme, dark green: tyrosyl ring deiodinations catalyzed by the respective Dio isozyme, black: deiodination reactions which were theoretically possible but found not to be catalyzed by the respective Dio isozyme.

3.5 Functional validation of newly identified TAM substrates

To substantiate the newly identified TAM deiodination reactions, it was investigated which TAMs would interfere with an established TH deiodination reaction. Those TAMs which had been identified as Dio substrates in the LC-MS/MS experiments were expected to compete with a classical TH substrate in a Dio catalyzed reaction. By contrast, those TAMs which were excluded as Dio substrates in the LC-MS/MS experiments were not expected to do so.

Mouse liver membrane fractions, which represent a rich source of Dio1 as demonstrated in Figure 12, were subjected to $^{125}I^-$ release assays using rT_3 as substrate and increasing concentrations of the various TAMs as inhibitors. Mouse liver membrane fractions were chosen as an exemplary Dio source in these assays since both phenolic and tyrosyl ring TAM substrates had been identified for this enzyme preparation in LC-MS/MS experiments (Figure 13). rT_3 was chosen since it represents the preferred substrate of murine Dio1. In line with previous reports, rT_3 was 5'-deiodinated by Dio1 from mouse liver membrane fractions following Michaelis-Menten kinetics with an apparent K_m of 0.43 ± 0.023 µM and apparent v_{max} of 142.7 ± 2.7 pmol $^{125}I^-$ released/mg/min (Figure 12).

rT_3AM, 3',5'-T_2AM and 3,3'-T_2AM, which had been identified as phenolic ring substrates of Dio1 from mouse liver membrane fractions in LC-MS/MS experiments, inhibited the 5'-deiodination of rT_3. Both 3',5'-T_2AM and 3,3'-T_2AM lead to a significant increase in apparent K_m as determined by Friedman test followed by Dunn's post test (Figure 24A, B; Table 18). Moreover, 3',5'-T_2AM and 3,3'-T_2AM caused slight decreases in apparent v_{max}, which reached statistical significance.

Therefore, 3',5'-T_2AM and 3,3'-T_2AM acted as mixed inhibitors, with competitive and noncompetitive elements. Dixon plots revealed K_i values of 2.6 ± 0.4 µM and 4.8 ± 0.1 µM for 3',5'-T_2AM and 3,3'-T_2AM, respectively (Figure 24A, B; Table 18). By contrast, rT_3AM decreased the apparent v_{max} of rT_3 conversion without significantly changing the apparent K_m indicating that rT_3AM behaved as a noncompetitive inhibitor (Figure 24C; Table 18). From the change in apparent v_{max} for rT_3, a K_i value of 1.3 ± 0.2 µM was calculated for rT_3AM.

T_3AM and 3,5-T_2AM, which had been identified as weak tyrosyl ring substrates of Dio1 (Figure 13C, F), also acted as noncompetitive inhibitors (Figure 24D, E; Table 18). As expected from the LC-MS/MS studies, T_3AM and 3,5-T_2AM exhibited high K_i values of 34.0 ± 2.6 µM and 85.0 ± 7.7 µM, respectively.

TAMs which were not converted by Dio1 from mouse liver membrane fractions in the LC-MS/MS experiments, namely T_4AM, 3'-T_1AM and 3-T_1AM, failed to show any effect on the 5'-deiodination of rT_3 by Dio1 up to a concentration of 10 µM (Table 18).

Taken together, the results of the competition assays were consistent with the data obtained from the LC-MS/MS experiments.

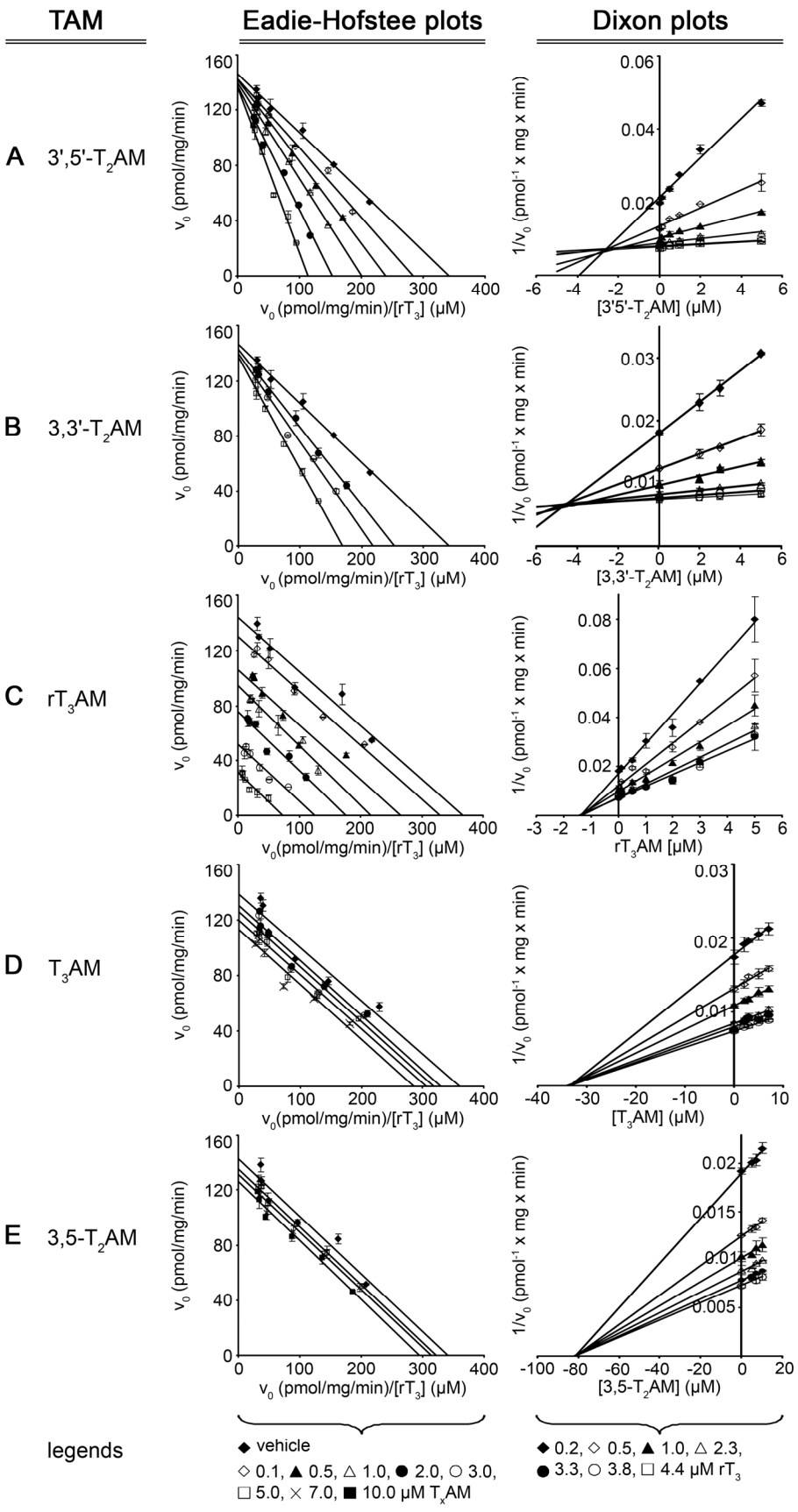

TAM
Eadie-Hofstee plots
Dixon plots
A 3',5'-T2AM
B 3,3'-T2AM
C rT3AM
D T3AM
E 3,5-T2AM
v0 (pmol/mg/min)
v0 (pmol/mg/min)/[rT3] (μM)
1/v0 (pmol-1 x mg x min)
[3'5'-T2AM] (μM)
[3,3'-T2AM] (μM)
rT3AM [μM]
[T3AM] (μM)
[3,5-T2AM] (μM)
legends
◆ vehicle
◇ 0.1, ▲ 0.5, △ 1.0, ● 2.0, ○ 3.0, □ 5.0, × 7.0, ■ 10.0 μM TxAM
◆ 0.2, ◇ 0.5, ▲ 1.0, △ 2.3, ● 3.3, ○ 3.8, □ 4.4 μM rT3

Figure 24: TAM substrates of Dio1 inhibit the 5'-deiodination of rT_3 by Dio1 in $^{125}I^-$ release assays. The effect of TAMs on the apparent K_m and v_{max} of the 5'-deiodination of rT_3 by Dio1 was analyzed using Friedman test followed by Dunn's post test and $p < 0.05$ was considered significant. 3',5'-T_2AM and 3,3'-T_2AM acted as mixed inhibitors (**A, B**) whereas rT_3AM, T_3AM and 3,5-T_2AM caused noncompetitive inhibition (**C - E**). The mean ± SD values reported for apparent K_m, apparent v_{max} and K_i values are from three separate experiments performed in triplicate.

Table 18: Effects of iodothyronamines on the 5'-deiodination of rT_3 by Dio1 from mouse liver membrane fractions.

	Apparent K_m (μM rT_3)	**Apparent v_{max} (pmol [a]/mg/min)**	**K_i (μM)**	**Mode of inhibition**
vehicle	0.43 ± 0.02	142.7 ± 2.7	na	na
3',5'-T_2AM (5 μM)	1.19 ± 0.1*	136.4 ± 4.8*	2.6 ± 0.4	mixed
3,3'-T_2AM (5 μM)	0.86 ± 0.04*	136.9 ± 2.9*	4.8 ± 1.1	mixed
rT_3AM (5 μM)	0.42 ±0.01	31.3 ± 2.3*	1.3 ± 0.2	noncompetitive
T_3AM (5 μM)	0.43 ± 0.03	124.5 ± 4.7*	34.0 ± 2.6	noncompetitive
3,5-T_2AM (5 μM)	0.42 ± 0.02	134.9 ± 2.1*	85.0 ± 7.7	noncompetitive
T_4AM (5 μM)	0.43 ± 0.03	144.2 ± 4.1	na	na
3'-T_1AM (5 μM)	0.42 ± 0.04	141.9 ± 1.4	na	na
3-T_1AM (5 μM)	0.43 ± 0.03	142.1 ± 5.2	na	na

The apparent K_m, apparent v_{max} and K_i values are from three separate experiments performed in triplicate and are presented as mean ± SD. * designates statistical difference with $p < 0.05$ as determined by using Friedman test followed by Dunn's post test. na: not applicable. [a] I^- released. Shading: light grey: TAMs which have been identified as phenolic ring substrates of Dio1 in LC-MS/MS assays, grey: TAMs which have been identified as tyrosyl ring substrates of Dio1 in LC-MS/MS assays, dark grey: TAMs which have been excluded as Dio1 substrates in LC-MS/MS assays.

3.6 Kinetic constants of selected TAM deiodination reactions

To characterize the kinetic properties of TAM deiodination reactions, the apparent K_m and v_{max} of selected TAM deiodination reactions were measured and compared to that of the corresponding TH.

At first, the deiodination of rT_3AM by all Dio preparations used herein was compared to that of rT_3. rT_3AM was chosen since it represented the only TAM which had been identified as a substrate of all Dio isozymes. Furthermore, it represented the TAM corresponding to the preferred TH substrate of human and murine Dio1, namely rT_3. For each Dio preparation used, the apparent K_m values for the deiodination of rT_3AM were comparable to those observed with rT_3 (Table 19). The 5'-deiodination of rT_3AM by Dio1 from HepG2 lysates and mouse liver membrane fractions as well as by Dio2 from MSTO-211H lysates was catalyzed at lower apparent v_{max} values compared to rT_3 (Table 19). By contrast, the apparent v_{max} of the 5-deiodination of rT_3AM by Dio3 from ECC-1 lysates was more than twofold higher compared to that observed for rT_3 (Table 19).

Secondly, the 5-deiodination of T_3AM by Dio3 from ECC-1 lysates was compared to that of T_3. T_3AM was chosen since it represented the TAM corresponding to the preferred substrate of Dio3, namely T_3. Both reactions were characterized by a similar apparent K_m, however, T_3AM was 5-deiodinated at a lower apparent v_{max} than T_3. For Dio2, the preferred substrate T_4 could not be compared to its corresponding TAM since T_4AM was excluded as a substrate of Dio2 from MSTO-211H lysates (see section 3.4.3).

Finally, the apparent K_m and v_{max} of the 3-deiodination of 3-T_1AM to T_0AM by Dio3 from ECC-1 lysates was studied since this reaction might directly account for the biosynthesis of T_0AM *in-vivo*. The reaction was catalyzed at an apparent K_m of 1.2 ± 0.3 µM and an apparent v_{max} of 2.4 ± 0.06 pmol T_0AM/mg protein/min. By contrast, 3-iodothyronine (3-T_1), which represents the corresponding iodothyronine, was deiodinated at the tyrosyl ring at an apparent K_m of 1.6 ± 0.09 µM and an apparent v_{max} of 0.089 ± 0.05 pmol T_0/mg protein/min. Hence, 3-T_1AM was 3-

deiodinated at a higher v_{max}/K_m ratio and thus represented a better substrate of Dio3 from ECC-1 lysates than 3-T_1 (Table 19; compare Figure 17H to 22F).

Table 19: Apparent K_m and v_{max} of selected TAM and TH deiodination reactions as measured by LC-MS/MS.

Dio isozyme	**Dio preparation**	**substrate**	**K_m (µM substrate)**	**v_{max} (pmol deiodination product/mg protein/min)**
Dio1	HepG2	rT_3AM	0.63 ± 0.05	1.5 ± 0.08
		rT_3	0.59 ± 0.03	2.2 ± 0.06
	mouse liver	rT_3AM	0.48 ± 0.04	83.4 ± 1.9
		rT_3	0.43 ± 0.02	142.7 ± 2.7
Dio2	MSTO-211H	rT_3AM	0.006 ± 0.002	0.088 ± 0.012
		rT_3	0.005 ± 0.001	0.130 ± 0.026
Dio3	ECC-1	rT_3AM	0.29 ± 0.03	0.42 ± 0.02
		rT_3	0.29 ± 0.04	0.18 ± 0.03
		T_3AM	0.017 ± 0.004	1.9 ± 0.2
		T_3	0.006 ± 0.002	2.9 ± 0.2
		3-T_1AM	1.2 ± 0.3	2.4 ± 0.06
		3-T_1	1.6 ± 0.09	0.089 ± 0.05

The apparent K_m and v_{max} values were determined by LC-MS/MS based Dios assays using an incubation time of 30 min and 10 nM - 5 µM of the respective TAM as substrate. The values represent the mean ± SD from three separate experiments performed in triplicate.

4 Discussion

4.1 Analysis of TAM and TH deiodination reactions LC-MS/MS

The central objective of this study was to analyze the ability of the Dio isozymes to accept TAMs as substrates. Such experiments required the development of a method for the detection and quantification of TAMs which had been subjected to Dio catalyzed reactions. However, non-radiolabeled TAMs were incompatible with all techniques which have so far been used to study Dio catalyzed reactions. Deiodination reactions have mainly been analyzed employing ^{125}I or ^{131}I radiolabeled substrates in iodide release assays, high performance liquid chromatography (HPLC) methods, thin layer chromatography protocols or radioimmuno assays (RIAs) (for detailed review see Köhrle, 02). The generation of TAM specific antibodies as a prerequisite to the analysis of TAMs by RIAs has been hampered by their small size and great similarity in structure. TAMs are a group of nine small molecules exhibiting molar masses between 230.3 and 733.9 g/mol [Scanlan, 04]. Moreover, they differ from one another only regarding the number or the position of the iodine atoms (Figure 5). Several TAMs represent constitutional isomers, such as T_3AM and rT_3AM or 3-T_1AM and 3'-T_1AM. Apart from being poorly accessible to antibody detection, the analysis of TAM deiodination reactions by any of the aforementioned radiochemical methods was hampered by the unavailability of ^{125}I or ^{131}I radiolabeled TAMs. So far, no method has been developed to study deiodination reactions of non-radioactively labeled substrates.

Mass spectrometric techniques allow for analyzing compounds without radioactive labeling, such as endogenous compounds. Although LC-MS and LC-MS/MS methods for the detection of various unlabeled Dio substrates, such as T_4 and T_3, have been developed [Gu, 07; Soldin, 05; Soukhova, 04; Holm, 04; Thienpont, 99; Tai, 04; Tai, 02; De Brabandere, 98], these techniques have not been used to study Dio catalyzed reactions but rather to investigate the concentrations of the respective THs in tissue or cell culture samples. Furthermore, although one LC-MS/MS method has been published for the detection of 3-T_1AM and T_0AM from

serum and solid tissues [Scanlan, 04], no protocol was available allowing for the analysis of the complete TAM panel including the discrimination of TAM constitutional isomers. However, in order to monitor potential sequential TAM deiodination reactions, it was necessary to include TAMs of all iodination grades. Furthermore, in order to clearly distinguish between phenolic and tyrosyl ring deiodination of a TAM substrate, it was essential to determine the exact constitutional isomerism of the product of the deiodination reaction. Therefore, an important first objective of this study was to establish an LC-MS/MS method capable of the unequivocal, simultaneous identification all TAMs in the same sample.

To evaluate the possible physiological relevance of putatively identified TAM deiodination reactions, the kinetic constants K_m and v_{max} should be determined and compared to that of established Dio catalyzed reactions, e.g. TH deiodination reactions. Therefore, the LC-MS/MS method should not only allow for the identification but also for the quantification of TAMs. However, so far, no reference method for TAM quantification has been available. Thus, a further objective of the present study was to include the complete panel of THs as established Dio substrates into the LC-MS/MS protocol. This would allow for validating the performance of the LC-MS/MS method in quantifying Dio catalyzed reactions by comparing the kinetic constants of TH deiodination reactions obtained by LC-MS/MS to that obtained by independent methods, such as $^{125}I^-$ release assays or HPLC based radiochemical assays.

In line with TH constitutional isomers, such as 3-T_1 and 3'-T_1 [Zhang, 06b] or 3,5-T_2, 3,3'-T_2 and 3',5'-T_2 [Zhang, 06a], constitutional TAM isomers were indistinguishable in terms of MS/MS parameters. Fragmentation of the parent ion in the second quadrupole yielded identical product ions even if the percent contribution of each product ion was unique to each constitutional isomer. This was observed both in the positive (Figure S28) and in the negative electrospray ionization mode (data not shown). Therefore, the distinction of constitutional TAM and TH isomers required their chromatographic separation prior to the detection by MS/MS. The novel HPLC method developed herein allowed for the complete baseline separa-

tion of all except one pair of constitutional TAM and TH isomers. This has not been achieved by any other chromatographic method, so far. Thus, the novel LC-MS/MS method presented herein, allowed for the unequivocal detection and quantification of all TAMs and THs from the same sample. Accordingly, the position of each iodine atom removed from a TAM or TH substrate in a Dio catalyzed reaction was backtracked unequivocally.

The novel LC-MS/MS method had further advantages over the classical radiochemical assays. First of all, it allowed for analyzing deiodination reactions of substrates without radioactive labeling. Thus, it circumvented a general disadvantage of all radiochemical assays, which is that they depend on the assumption that the isotopic substitution of an atom has no effects on the chemical properties of the substrate and that any effects on its kinetic properties are small enough to be neglected in the analysis [Cornish-Bowden, 04]. Being independent from radioactive labeling, a broader spectrum of candidate substrates could be investigated by LC-MS/MS. Secondly, employing the novel LC-MS/MS method product formation and substrate disappearance were monitored simultaneously and were both quantifiable. Moreover, sequential monodeiodinations were monitored. By contrast, iodide release assays fully rely on the equimolar production of labeled iodide and deiodinated product. Thus, using such assays, it remains elusive if the removal of the labled iodine atom from the substrate was preceded by the removal of an unlabeled iodine atom or by an alternate metabolization reaction of the substrate. Analyzing deiodination reactions of ^{125}I or ^{131}I radiolabeled substrates by chromatographic techniques yields a more comprehensive picture of the reaction since both the labeled substrate and the labeled product(s) are detected. However, once the ^{125}I or ^{131}I label has been cleaved off, the further fate of the deiodination product remains elusive. Finally, in contrast to RIAs, LC-MS/MS experiments are devoid of antibody cross reactions. Compared to radioactive assays, this LC-MS/MS method was of course less sensitive and more time-consuming but yielded a more direct and complete picture of deiodination cascades, which was considered indispensable for establishing new enzyme substrates.

The LC-MS/MS based analysis of TAMs and THs from Dio reaction mixtures required their prior extraction by a method compatible with LC-MS/MS measurements. Thus, a method based on liquid-liquid extraction allowing for the simultaneous isolation of all TAMs and THs from Dio reaction mixtures was developed herein (section 2.3). This method did not produce any interfering effects for the detection of all analytes with Dio reaction matrices (section 3.2.2). Furthermore, it yielded reproducible recovery rates, which were independent from the protein content, pH and amount of DTT of the Dio reaction matrix as well as from the addition of PTU (section 3.2.4). However, the recovery rates of TAM and TH analytes containing one or two iodine atoms were higher than that of the remaining analytes (Figure 9). Accordingly, the former analytes yielded lower LOD and LOQ values compared to the latter analytes (Table 16). Furthermore, in terms of LOD and LOQ values, the liquid-liquid extraction and LC-MS/MS detection protocol was more sensitive for TAM analytes when compared to the respective corresponding TH analytes (compare for instance right panel of Figure 11 to right panel of Figure 19). This was not due to differences in recovery rates (Figure 9). However, the more sensitive detection of TAMs compared to THs had already been observed in chromatograms of standard solutions which had not been subjected to deiodination reactions and liquid-liquid extraction (Figure 7C). Therefore, it might be postulated that the conditions used for HPLC and analyte ionization were more effective in producing $(M+H)^+$ ions of analytes with ethylamine side chains than $(M+H)^+$ ions with β-alanine side chains.

4.2 Isozyme specificity of Dio preparations

In order to study the substrate specificity of each Dio isozyme towards TAMs, it was essential to generate isozyme specific Dio preparations. Since the LC-MS/MS studies required high specific activities of each Dio isozyme, endogenously expressed enzymes were preferred over transfected and recombinantly expressed ones. The cell lines used in this study were well-established isozyme specific sources for the respective Dio and had already been used as isozyme

specific Dio models by other research groups: HepG2 for Dio1 [Kester, 06; Jakobs, 02], MSTO-211H for Dio2 [Curcio, 01] and ECC-1 for Dio3 [Kester, 06]. Furthermore, membrane fractions from murine livers have frequently been described as a rich source of Dio1 [Schoenmakers, 92; Schneider, 01; Schneider, 06; Streckfuss, 05]. Nevertheless, several experiments of the present study aimed at verifying the exclusive expression of the respective Dio isozyme in each Dio preparation. However, characterizing the expression of Dio isozymes in cell lines and tissues is challenging in several respects.

First of all, Dio isozymes are representatives of selenoproteins [Köhrle, 05]. The insertion of the rare amino acid selenocysteine (Sec) into the active center of Dio isozymes occurs cotranslationally at a UGA stop codon whose recoding requires the concerted interaction of the Sec-loaded $tRNA^{Ser(Sec)}$, the Sec insertion sequence (SECIS) and several mRNA and tRNA binding proteins [Köhrle, 05]. Successful selenoprotein translation is very complex, error-prone and less efficient than that of typical proteins [Gereben, 02; Dumitrescu, 05; Berry, 91b]. Thus, the presence of a Dio transcript does not inevitably guarantee its translation into a functional protein [Köhrle, 05]. Therefore, when measuring the Dio transcripts in candidate cell lines and tissues by RT-PCR, cycle numbers within and beyond the log-linear range were used (section 2.7.6) since it was more important to exclude the expression of individual Dio mRNAs in a cell line or tissue than to (semi)quantify the concentrations of the detectable Dio mRNAs. Accordingly, the presence of any Dio1 and Dio3 transcript in MSTO-211H cells was unequivocally excluded thus validating the isozyme specificity of MSTO-211H cells for Dio2 (Figure 14A). Likewise, the presence of Dio3, Dio2 and Dio1 transcripts in HepG2 cells, mouse liver and HEK293 cells was ruled out, respectively (Figure 10A, 12A, 18A). However, the Dio2 transcript, which was detected in all cell lines, only corresponded to Dio2 enzymatic activity in MSTO-211H cells but was not functional in the other cell lines. Similarly, the Dio3 transcript detected in mouse liver and HEK293 cells did not correlate to any Dio3 enzymatic activity (Figure 12, 18).

A second difficulty encountered when analyzing the expression of Dio isozymes is the unavailability of antibodies specific for Dio isozymes. Therefore, studies characterizing Dio proteins usually employ non-immunological techniques, such as affinity labeling with alkylating substrates analogs, such as N-bromoacetyl-[^{125}I]-T_3/T_4, or ^{75}Se incorporation. However, these methods do not allow for the discrimination between Dio isozymes, either.

Thus, the only way to comprehensively characterize the expression of Dio isozymes in cell lines or tissues is to analyze both the Dio transcripts and the cognate enzymatic activities. In this study, the enzymatic Dio activities were not merely measured using one established substrate of the respective isozyme. Instead, by taking advantage of the novel LC-MS/MS method, the substrate specificity of the Dio preparations towards all iodothyronines was analyzed and compared to literature data. The ability of the LC-MS/MS method to backtrack the position of each iodine atom removed from the substrate combined with the usage of the Dio1 specific inhibitor PTU allowed for unequivocally assigning deiodination reactions to Dio isozyme identities (see sections 1.2.1 - 1.2.3). Phenolic and tyrosyl ring deiodination reactions which were insensitive to PTU were attributed to Dio2 and Dio3, respectively. By contrast, both phenolic and tyrosyl ring deiodination reactions which were sensitive to PTU were classified as Dio1 catalyzed reactions.

Both HepG2 lysates and mouse liver membrane fractions catalyzed a phenolic ring deiodination of T_4 yielding T_3 and a tyrosyl ring deiodination of T_4 yielding rT_3, which was almost completely 5'-deiodinated to 3,3'-T_2 (Figure 11A, 13A). Furthermore, both Dio1 preparations catalyzed deiodinations of rT_3 and 3',5'-T_2 at their phenolic rings. As these reactions were completely inhibited by PTU, they were clearly catalyzed by Dio1. These substrate specificities and inhibition characteristics were in line with published properties of Dio1 [Köhrle, 02; Kuiper, 05; Lumholtz, 78]. However, only mouse liver membrane fractions but not HepG2 lysates catalyzed the phenolic ring deiodination of 3,3'-T_2 and the tyrosyl ring deiodinations of T_3 and 3,5-T_2, all of which have frequently been described as Dio1

catalyzed reactions [Kuiper, 05; Wu, 05; Chopra, 82]. Therefore, both Dio1 preparations were used to study the ability of Dio1 to accept TAMs as substrates.

MSTO-211H lysates catalyzed the phenolic ring deiodinations of all THs possessing two iodine atoms in the phenolic ring, namely T_4, rT_3 and 3',5'-T_2. Since these reactions were not inhibited by 1 mM PTU, they were unambiguously catalyzed by Dio2. However, so far, only T_4 and rT_3 have been described as Dio2 substrates [Köhrle, 02] whereas no study has been published analyzing 3',5'-T_2 as a substrate of Dio2 (Figure 4). Considering the absence of Dio1 and Dio3 transcripts in MSTO-211H cells, which validated this cell line as an exclusive source of Dio2, 3',5'-T_2 was considered as a newly identified substrate of Dio2. The remaining THs possessing one iodine atom at their phenolic ring, namely T_3, 3,3'-T_2 and 3'-T_1, which have either previously been excluded as Dio2 substrates or not been reported as Dio2 substrates yet [Chopra, 81; Smallridge, 84] (Figure 4), were not converted by MSTO-211H lysates. Taken together, MSTO-211H lysates were considered as an isozyme specific source of Dio2 capable of deiodinating all known TH substrates of Dio2 and therefore appropriate to comprehensively analyze the substrate specificity of Dio2 towards TAMs as a new group of potential Dio substrates.

ECC-1 lysates had previously been described as an isozyme specific source of Dio3 [Kester, 06]. However, when analyzing their substrate specificity towards THs both in the presence and in the absence of 1 mM PTU, two types of deiodination reactions were observed. Firstly, all THs possessing iodine atoms in their tyrosyl rings were 5- or 3-deiodinated in a PTU-insensitive fashion (Figure 22). Secondly, T_4, rT_3 and 3',5'-T_2 were weakly deiodinated at their phenolic rings, but those reactions were inhibited by PTU (data not shown). In line with these LC-MS/MS experiments, analysis of ECC-1 lysates by $^{125}I^-$ release assays employing rT_3 as substrate, disclosed a weak PTU sensitive enzymatic Dio1 activity (specific activity of 23 ± fmol/mg protein/min at a final rT_3 concentration of 1 µM). Thus, the phenolic ring deiodination reactions of T_4, rT_3 and 3',5'-T_2 and were attributed to Dio1. Furthermore, in all following experiments, 1 mM PTU was added to each

reaction catalyzed by ECC-1 lysates to efficiently inhibit the Dio1 enzymatic activity. Of all tyrosyl ring TH substrates identified for Dio3 from ECC-1 cells in the present study, T_4, rT_3, T_3, 3,3'-T_2 and 3,5-T_2 had been described before [Köhrle, 02; Sorimachi, 79a; Sorimachi, 77]. By contrast, 3-T_1, which has not been reported as a substrate of Dio3, so far, was identified as a new but weak Dio3 substrate in the present study.

Taken together, the position of the iodine atoms removed from TH substrates was always, without any exception, in line with the published Dio catalytic properties. With Dio1 preparations both phenolic and tyrosyl ring deiodination reactions were observed whereas phenolic and tyrosyl ring deiodination reactions were observed exclusively with Dio2 and Dio3 preparations, respectively. Additionally, all TH substrates previously described were indeed converted by the respective Dio isozymes. These findings confirmed the isozyme specificity of the Dio preparations used in this study.

4.3 Dio substrate specificity towards TAMs

The LC-MS/MS screening experiments demonstrated that all Dio isozymes were capable of catalyzing TAM deiodination reactions. Each Dio isozyme exhibited a unique substrate specificity towards TAMs. Dio3 from ECC-1 lysates displayed the broadest substrate specificity since it accepted all TAMs with tyrosyl ring iodine atoms as substrates. By contrast, the substrate specificities of Dio1 and Dio2 were more restricted. Dio2 from MSTO-211H lysates catalyzed only phenolic ring deiodinations of rT_3AM and 3',5'-T_2AM.

In the present study two different Dio1 preparations, namely HepG2 lysates and mouse liver membrane fractions were used. Interestingly, their substrate specificities did not overlap completely. While rT_3AM and 3',5'-T_2AM were deiodinated at the phenolic rings by both preparations, 3,3'-T_2AM, T_3AM and 3,5-T_2AM were exclusively deiodinated by mouse liver membrane fractions (compare Figure 19 to Figure 20). These differences were in line with the discrepancies observed in the

studies analyzing the substrate specificities of HepG2 lysates and mouse liver membrane fractions towards THs. While T_4, rT_3 and 3',5'-T_2 were deiodinated at the phenolic rings by both preparations, T_3, 3,3'-T_2 and 3,5-T_2 were exclusively converted by mouse liver membrane fractions (compare Figure 11 to Figure 13). These discrepancies persisted even when the pH of the homogenization buffer and the potassium phosphate buffer were progressively increased to 8.0 (refer to Table 11, data not shown). Therefore, these data might suggest that species-specific factors modulate the Dio1 substrate specificity towards TAMs. This notion was supported by the finding that the weak Dio1 enzymatic activity detected in the human ECC-1 cell line catalyzed the phenolic ring deiodination of T_4, rT_3 and 3',5'-T_2 but no PTU sensitive deiodinations of T_3, and 3,3'-T_2 and 3,5-T_2 (data not shown). So far, no species-specific substrate specificities have been reported for Dio isozymes. However, species-specific substrate preferences have been reported for Dio1, which in dogs prefers sulfated T_3 and sulfated 3,3'-T_2 over rT_3 but in humans, rats and mice prefers rT_3 over other (sulfated) THs [Laurberg, 82; Schoenmakers, 92; Toyoda, 95; Toyoda, 97].

The identified TAM substrates were functionally verified by inhibiting the Dio1 catalyzed phenolic ring deiodination of rT_3, which represents the preferred deiodination reaction catalyzed by murine Dio1 [Köhrle, 02]. This experimental strategy has frequently been used to verify or even identify Dio substrates [Chopra, 77; Kaplan, 78; Visser, 79; Sorimachi, 79b; Fekkes, 82; Chopra, 82; Köhrle, 86]. For instance, in one study the phenolic ring deiodination of rT_3 by Dio1 from rat liver microsomes was competitively inhibited by THs which also serve as Dio1 substrates, namely 3',5'-T_2, T_4, T_3 and 3,5-T_2. By contrast, 3-T_1 and T_0, which do not represent Dio1 substrates, did not inhibit the reaction significantly [Fekkes, 82]. In the competition experiments performed herein, mouse liver membrane fractions were chosen as an exemplary Dio source for several reasons. First of all, mouse liver membrane fractions served as a rich and isozyme specific source of Dio1. Secondly, they represented a Dio1 preparation of high physiological and *ex-vivo* relevance. Finally, both phenolic and tyrosyl ring TAM deiodination reactions were identified for this enzyme preparation in the LC-MS/MS based Dio assays (Fig-

ure 20). In the competition experiments, the phenolic ring substrates rT_3AM, 3',5'-T_2AM and 3,3'-T_2AM were potent inhibitors exhibiting K_i values in the lower µM range, i.e. comparable to the K_m value of the phenolic ring deiodination of rT_3 of roughly 0.4 µM (Table 18). By contrast, the K_i values of the tyrosyl ring substrates T_3AM and 3,5-T_2AM were roughly one order of magnitude higher. These data were consistent with the LC-MS/MS experiments in which the phenolic and tyrosyl ring substrates were converted readily and weakly, respectively. Furthermore, TAMs which had been excluded as substrates of Dio1 from mouse liver membrane fractions in the LC-MS/MS studies, namely T_4AM, 3'-T_1AM and 3-T_1AM, failed to show any effect in the competition assays thus verifying the former results.

It is unclear why only some TAM substrates acted as mixed inhibitors with predominantly competitive elements (3',5'-T_2AM and 3,3'-T_2AM) whereas other TAMs unexpectedly acted as noncompetitive inhibitors (rT_3AM, T_3AM and 3,5-T_2AM; see Table 18) thus suggesting a more complex mechanism of interference than mere competition of two substrates for the same active center. So far, important data are lacking which might help to clarify this discrepancy. First of all, the number of active centers of the endogenous Dio1 holoenzyme remains elusive [Leonard, 05]. The finding that Dio1 catalyzes both phenolic and tyrosyl ring deiodination reactions, even with the same substrate, namely T_4, has already led to the hypothesis that the Dio1 enzyme might possess more than one active center. Different modalities for substrate binding were suggested so that either the iodine atoms of the phenolic ring of those of the tyrosyl ring are in close proximity to the active center of the enzyme [Toyoda, 97]. Either two distinct binding sites for phenolic and tyrosyl ring deiodination substrates or a single site to which substrates bind with a considerable degree of freedom or in different orientations were proposed [Toyoda, 97]. Supporting a single active center, it was demonstrated that the Dio1 mRNA encodes a 27 kD polypeptide, transfection of the cognate cDNA yielded a catalytically active Dio1 [Berry, 91a], and the 27 kD polypeptide functioned as an independent catalytic center [Curcio-Morelli, 03]. However, two 27 kD polypeptides were reported to homodimerize [Leonard, 01], and catalytic

activity was preserved when only one subunit was catalytically competent [Leonard, 05]. The three-dimensional structure of Dio1, which might help to determine the number of active centers of the enzyme, has not been resolved yet. In addition to uncertainties about the composition of the Dio1 holoenzyme, the molecular conformation of only one TAM, namely T_3AM, has been reported [Cody, 84]. Therefore, comprehensive structure-function relationships for Dio1 and TAM substrates, comparable to those of Dio1 and TH substrates, are not available. Speculations on inhibition mechanisms based on extrapolations from the available structures of THs seems daring, as the presence (in THs) or absence (in TAMs) of the dissociated carboxylate functional group in the tyrosyl ring side chain might contribute to different conformational orientations of the iodothyron(am)ines with iodine atoms in 3- and 5-position, which limits the flexibility of the phenolic ring as illustrated by the skewed or anti-skewed structures reported for THs [Cody, 80; Cody, 81; Okabe, 82]. However, so far, it can be excluded that the modes of inhibition depend solely on the iodine substitution pattern of the TAM substrates on the position of the iodine removed.

4.4 Substrate preferences and kinetic properties of deiodination reactions

The capability of the LC-MS/MS detection method to monitor sequential deiodination reactions provided a tool to estimate the substrate preferences of the Dio isozymes. Incubation of HepG2 lysates and mouse liver membrane fractions with T_4 revealed that T_4 was weakly 5'-deiodinated to T_3 (Figure 11A; Figure 13A), which is consistent with previous findings [Chopra, 76; Visser, 78; Kaplan, 78]. Furthermore, a small 3,3'-T_2 peak was observed. Theoretically, 3,3'-T_2 might have been formed by further 5-deiodination of T_3 or by 5-deiodination of T_4 to rT_3 and subsequent rapid 5'-deiodination of rT_3. The latter interpretation is consistent with published data [Visser, 78; Visser, 79] and supported by the finding that rT_3 was almost completely 5'-deiodinated to 3,3'-T_2 by Dio1 preparations whereas T_3 was

only weakly 5-deiodinated by mouse liver membrane fractions or not deiodinated at all by HepG2 lysates (Figure 11B, C; Figure 13B, C). Thus, without determining the kinetic constants of T_4, T_3 and rT_3 conversion by Dio1, it can be concluded at least qualitatively that human and murine Dio1 prefer rT_3 over T_4 and T_3, which is in line with published substrate preferences [Köhrle, 02].

Interestingly, further sequential monodeiodination cascades were only observed in the cases of the tyrosyl ring deiodinations of T_3 and T_3AM by Dio3 containing ECC-1 lysates (Fig. 17C, Figure 22C). The sequential monodeiodination of T_3 has already been reported using rat brain homogenates [Kaplan, 80] or intact cultured NCLP-6E monkey hepatocarcinoma cells as sources of Dio3 [Sorimachi, 77]. However, in all other experiments described herein, one-step deiodination reactions were monitored even if the product proved to be readily convertible by the respective Dio isozyme when used as a direct substrate. Considering that the sensitivity of the LC-MS/MS method used herein was inversely proportional to the degree of iodination of TAM and TH analytes, except for the more sensitive detection of T_1(AM)s compared to T_0(AM), the predominant absence of sequential monodeiodination reactions indicates that Dio isozymes prefer TAMs and THs with higher iodine content over those with lower iodine content as substrates.

In previous publications, further sequential deiodination cascades have been described. For instance, in one study using intact NCLP-6E monkey hepatocarcinoma cells, which express both Dio1 and Dio3 [Sorimachi, 79b; Baqui, 03], [3',5'-^{125}I]T_4 was sequentially deiodinated at the phenolic ring by Dio1 yielding rT_3 and 3',5'-T_2. Furthermore, the T_3 formed from T_4 was completely deiodinated at the tyrosyl ring by Dio3 yielding 3,3'-T_2 and 3'-T_1. Additionally, sulfated 3,3'-T_2 and sulfated 3'-T_1 were detected [Sorimachi, 79a]. However, since T_4 was metabolized by intact cultured cells, the cited study cannot be compared directly to this study which used Dio preparations, such as cell line homogenates and mouse liver membrane fractions. In intact cells but not in Dio preparations, the bioavailability of THs (and presumably also TAMs) to the active center of a Dio isozyme is regulated by their transport into the cell, their retention time within the cell and

non-deiodinative pathways, which might precede the deiodination reaction [Sorimachi, 79a]. Accordingly, several TH substrates were extensively sulfated by intact NCLP-6E cells [Sorimachi, 77] whereas little or no conjugation was observed with homogenates of this cell line [Sorimachi, 79b]. Likewise, rT_3 was sulfated by intact cultured rat hepatoma R117-21B cells but not by homogenates thereof [Sorimachi, 80b]. In line with these findings, the TAM and TH substrates were only subject to deiodination reactions but not to non-deiodinative pathways with the cell line lysates and mouse liver membrane fractions used in this study (section 3.3.1). Additionally, deiodination reactions catalyzed by Dio preparations, such as cell line homogenates, cannot automatically be extrapolated to *in-vivo* reactions observed in cultured cells or animal models because of the greatly supraphysiological thiol concentrations used [Kaplan, 83].

Despite the capability of the LC-MS/MS method to unravel relative substrate specificities among substrates and products of sequential monodeiodination cascades, kinetic experiments were also included in the present study. The apparent K_m and v_{max} of selected TAM deiodination reactions were measured and compared to that of the respective TH in order to estimate their physiological potential to be substrates of Dio isozymes. Considering the use of mainly cell line derived Dio preparations and the few data available on TAM concentrations *in-vivo*, the data obtained here are of preliminary nature. However, all TAM deiodination reactions analyzed quantitatively herein were catalyzed at apparent K_m values which were in the same concentration range as those observed with the respective corresponding TH [Köhrle, 02] (Table 19). These findings support a putative *in-vivo* relevance of the respective TAM reactions analyzed. Furthermore, the tyrosyl ring deiodinations of rT_3AM and $3\text{-}T_1AM$ by Dio3 from ECC-1 lysates exhibited higher apparent v_{max}/K_m ratios than their corresponding THs. Thus, with the Dio3 preparation and the reaction conditions used in this study, rT_3AM and $3\text{-}T_1AM$ represented better substrates of Dio3 than rT_3 and $3\text{-}T_1$.

4.5 Structural requirements of Dio substrates

The identification of TAMs as Dio substrates provided further new insights into the structural requirements for Dio substrates in general. Dio isozymes have long been known not only to convert THs but also various endogenous iodine containing TH metabolites [Wu, 05; Visser, 94; Köhrle, 84].

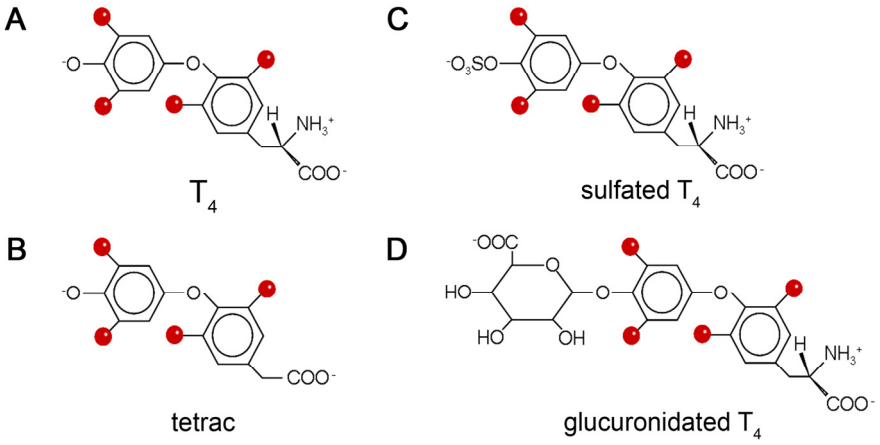


Figure 25: Structure of endogenous T_4 metabolites.

For instance, the glucuronic acid ester of T_4 (Figure 25D) was shown to be deiodinated at the phenolic ring by microsomes from euthyroid rat livers, which served as a source for Dio1, and by microsomes from hypothyroid rat brains, which were considered as a source for Dio2 [Hays, 92]. Furthermore, the acetic acid analogs to T_4 and T_3, namely tetraiodothyroacetic acid (tetrac) (Figure 25B) and triiodoacetic acid (triac) proved to be better substrates for hepatic Dio1 than T_4 and T_3 [Wu, 05; Köhrle, 84; Köhrle, 86]. Likewise, sulfation of the phenolic hydroxyl group of T_3 enhanced its tyrosyl ring deiodination by hepatic Dio1 [Visser, 94]. This facilitated deiodination upon sulfation was hypothesized to mechanistically be due to an improved interaction of the negatively charged sulfate group with protonated residues in the active center of the basic Dio1 protein [Visser, 94]. In comparison to THs, their glucuronidated and sulfated metabolites as well as their acetic acid analogs either carry additional negative charges or exhibit a nega-

tively charged tyrosyl ring side chain (Figure 25). However, so far, it has not been possible to generally attribute the enhanced catalytic Dio activities towards the aforementioned metabolites to their additional net negative charges. Instead, upon sulfation, the tyrosyl ring deiodinations of T_4, T_3 and triac by Dio1 are accelerated while the phenolic deiodinations may either be inhibited (T_4), unaffected (rT_3) or stimulated (3,3'-T_2, 3,3'- diiodothyroacetic acid) [Visser, 94].

The structural requirements of Dio substrates have further been studied extensively using large series of TH analogs [Köhrle, 84; Köhrle, 86]. However, apart from T_4AM, no other TAM was included in these studies. In one series of L-T_4 analogs [Köhrle, 84], highest affinities in terms of lowest apparent K_m values were obtained for substrates carrying a negatively charged but no positively charged functional group within the tyrosyl ring side chain (Figure 26E, F). Medium affinities were observed with L-T_4 and D-T_4, which both exhibit a zwitterionic side chain (Figure 26A, B), while lowest affinities were obtained for analogs with a positively charged side chain, such as T_4AM and L-T_4-ethylester (Figure 26C, D).

	Dio1 substrate	side chain structure	side chain charge	apparent K_m (µM)
A	L-T_4	CH_2-CH-COO^- NH_3^+	+/-	2 - 4
B	D-T_4	NH_3^+ CH_2-CH-COO^-	+/-	1 - 2
C	L-T_4AM	CH_2-CH_2 NH_3^+	+	nd
D	L-T_4-ethylester	CH_2-CH-COO^-CH_2-CH_3 NH_3^+	+	3.0
E	tetrac	CH_2-COO^-	-	0.2
F	N-acetyl-L-T_4	CH_2-CH-COO^- NH-C-CH_3 O	-	0.2

Figure 26: Structure-activity relationships of L-T_4 analogs for the phenolic ring deiodination catalyzed by Dio1 from rat liver microsomes. Partly adopted from [Köhrle, 84]. tetrac: tetraiodothyroacetic acid, nd: not detectable.

In the study cited above, the negative net charge of the tyrosyl ring side chain seemed to be dominant in determining the affinity of the T_4 analogs to Dio1, since tetrac and N-acetyl-L-T_4 exhibited similarly low K_m values despite great differences in side chain constitution and spatial confirmation (Figure 26 E, F). By contrast, N-acetyl-L-T_4 exhibited a much lower K_m value for Dio1 than L-T_4-ethylester (Figure 26D, F), a compound with a comparable bulky but positively charged side chain. [Köhrle, 84].

However, the data obtained in the present study do not confirm a dominant role of the net negative charge of the tyrosyl ring side chain in determining the affinity of a substrate to Dio1. Instead, in most instances, the TAM deiodinating reactions identified in this study were identical to the deiodination reactions observed with the corresponding THs (compare Figure 4 to Figure 23). First of all, Dio3 from ECC-1 lysates catalyzed tyrosyl ring deiodinations of all TAMs and THs possessing tyrosyl ring iodine atoms. Thus, at least with TAM and TH substrates, the sub-

strate specificity of Dio3 appears to be independent from the degree of iodination and the presence of the carboxylate group of the tyrosyl ring side chain. Secondly, both rT_3AM and rT_3 were readily converted by each Dio isozyme. Furthermore, 3',5'-T_2AM and 3',5'-T_2 were substrates of Dio1 and Dio2. Finally, both T_3AM, 3,3'-T_2AM, 3,5-T_2AM and their corresponding THs were deiodinated identically by Dio1 from mouse liver membrane fractions. Consequently, the specificity of the Dio isozymes towards the aforementioned substrates was independent from the tyrosyl ring carrying an ethylamine or a β-alanine side chain at the tyrosyl ring.

However, with T_4 and T_4AM the substrate specificity of the Dio isozymes towards TAMs and THs exhibited qualitative differences. While T_4 was accepted as a substrate of each Dio isozyme, T_4AM was identified as an exclusive substrate of Dio3. Hence, towards T_4 and T_4AM, the substrate specificity of the respective Dio isozyme might depend on the structure of the tyrosyl ring side chain. However, it can be excluded that the different acceptabilities of T_4 and T_4AM as Dio substrates are due to T_4 and T_4AM undergoing non-deiodinative metablization reactions which precede the deiodinations since it was demonstrated in preliminary experiments that, with the conditions used herein, the TAM and TH substrates were only subject to deiodination reactions (section 3.3.1).

The identification of TAMs as Dio substrates shows that Dio isozymes can convert endogenous substrates with a positively charged tyrosyl ring side chain. As mentioned above (section 4.4), some TAMs represented better Dio substrates than the corresponding TH, which has to be attributed to the positively charged ethylamine side chain.

4.6 The role of Dio isozymes in TAM biosynthesis

The identification of TAMs as isozyme specific Dio substrates strongly supports a role of Dio isozymes in the yet unknown pathways of TAM biosynthesis. There

are two putative mechanisms of TAM production, none of which can be excluded yet.

First of all, TAMs might be synthesized *de-novo*. This would require ether-bond coupling of two tyrosyl rings resembling the biosynthesis of T_4 and T_3 within the Tg molecule [Dunn, 01] Figure 1). Moreover, *de-novo* biosynthesis of 3-T_1AM from T_0AM would necessitate an iodination reaction of T_0AM. So far, such reactions have only been described within the thyroid gland involving TPO and DUOX [Ris-Stalpers, 06]. However, neither significant release of iodothyronines with lower iodination grade than T_4 or T_3 nor direct secretion of TAMs from the thyroid gland have been reported yet.

Secondly, TAMs could be derived from THs by decarboxylation of the β-alanine side chain. The aromatic amino acid decarboxylase (AADC) has been proposed as a candidate enzyme for TH decarboxylation [Scanlan, 04] partly due to its relatively broad substrate specificity including L-3,4-dihydroxyphenylalanine (L-dopa) and 5-hydroxytryptophane [Zhu, 95]. If there were decarboxylating activities for all THs, e.g. if 3-T_1 could be decarboxylated to 3-T_1AM and T_0 could be decarboxylated to T_0AM, the role of Dio isozymes in TAM biosynthesis would be an indirect one, namely synthesizing sufficient amounts of 3-T_1 and T_0 from T_4. But if the putative decarboxylating activities were restricted to iodine containing THs, i.e. 3-T_1 could be decarboxylated to 3-T_1AM but T_0 could not be decarboxylated to T_0AM, a tyrosyl ring Dio activity would be required directly to produce T_0AM from 3-T_1AM (Figure 27).

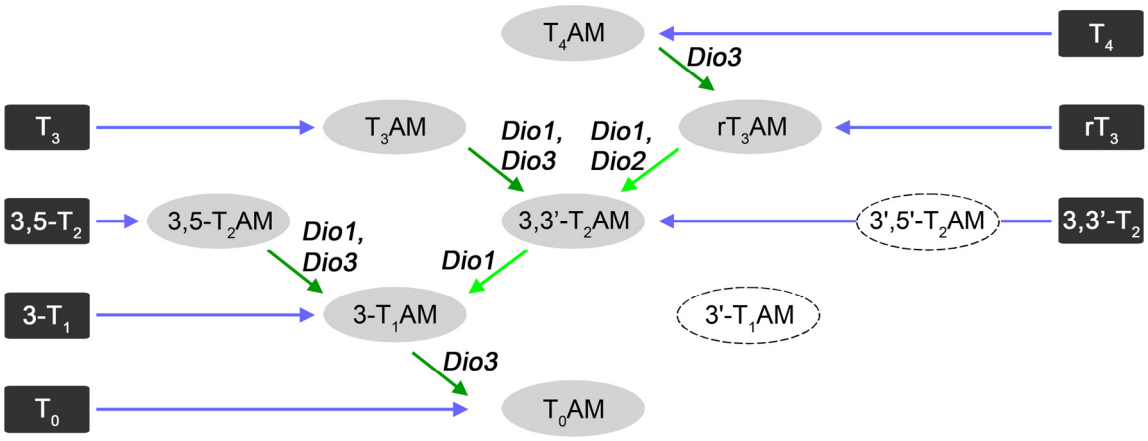


Figure 27: Pathways suggested for TAM biosynthesis. Arrows: light green: phenolic ring deiodination reactions catalyzed by Dio1 or Dio2, dark green: tyrosyl ring deiodination reactions catalyzed by Dio1 or Dio3. Blue arrows: putative decarboxylation reactions which might represent the first step of TAM biosynthesis. Dashed lines: TAMs which have been excluded as precursors of the biosynthesis of 3-T_1AM and T_0AM.

With the panel of newly identified TAM deiodinating reactions described here, sequential deiodination from T_4AM to T_0AM appears possible (Figure 27). Since a considerable number of deiodination reactions was excluded, this panel allows for confining the biosynthetic pathways of 3-T_1AM and T_0AM. It suggests a synthesis of endogenous T_0AM from 3-T_1AM via tyrosyl ring deiodination by Dio3 rather than from 3'-T_1AM via phenolic ring deiodination. By contrast, endogenous 3-T_1AM is equally likely to be synthesized from 3,3'-T_2AM via phenolic ring deiodination and from 3,5-T_2AM via tyrosyl ring deiodination, provided that 3,5-T_2AM or 3,3'-T_2AM occur *in-vivo*. So far, only 3-T_1AM and T_0AM have been detected endogenously [Scanlan, 04]. The detection of TAMs with higher iodine content *in-vivo* might have been hampered by the fact that their mass spectrometric detection is technically more difficult. Alternatively or additionally, their levels *in-vivo* could be very low due to putative rapid deiodination to 3-T_1AM and T_0AM. If the biosynthesis of T_0AM started with a decarboxylation of 3-T_1 or 3,5-T_2, it might be completed by Dio3 catalyzed reactions. However, if it started with a decarboxylation of 3,3'-T_2, T_3, rT_3 or T_4, both Dio1 and Dio3 would be required to complete

T_0AM biosynthesis. In the case of rT_3 or T_4 being the precursor of TAM biosynthesis, Dio2 activity might be involved to complete T_0AM biosynthesis catalyzing the phenolic ring deiodination of rT_3AM to 3,3'-T_2AM. However, this reaction might equally well be catalyzed by Dio1. Based on the newly identified substrate specificities of Dio isozymes towards TAMs, 3',5'-T_2AM and 3'-T_1AM are excluded as precursors of 3-T_1AM and T_0AM biosynthesis, respectively (Figure 27).

Considering the limited knowledge on the physiologic function(s) of TAMs and their concentrations *in-vivo* and, it is speculative to predict the tissues which might be involved in TAM biosynthesis.

4.7 Sulfated TAMs

Due to the recent discovery of TAM sulfation by hepatic, cardiac and brain sulfotransferases (SULTs), the spectrum of Dio substrates could become even broader [Pietsch, 07]. T_0AM and 3-T_1AM, which are detectable *in-vivo*, were found to be readily sulfated by human liver SULT1A3. Moreover, 3-T_1AM was sulfated by homogenates of human brain and cardiac tissue, i.e. target tissues of TAM action. The biological impact of TAM sulfation reactions remains to be established. The introduction of a sulfate group into a phenolic ring modifies its electronic environment [Wu, 05]. With many other endogenous and xenobiotic substrates, biological SULTs functions are inactivation and detoxification. Therefore, the sulfation of TAMs might serve to attenuate and thus regulate TAM actions [Pietsch, 07]. However, TAMs might also temporarily be inactivated by sulfation and be recovered from their sulfated form by the catalytic activity of sulfatases when TAM actions are required [Wu, 05]. In this respect, sulfated TAMs might serve as a reservoir for biologically active TAMs.

It remains to be tested, if sulfated T_0AM (T_0AMS), 3-T_1AMS and T_3AMS are detected *in-vivo*, whether they can also be deiodinated and, if so, whether TAM sulfation has any effect on the efficiency of their deiodination. According to literature data on TH sulfation, Dio1 would be the most likely candidate to catalyze deiodi-

nations of sulfated TAMs as deiodinations of sulfated THs by Dio2 and Dio3 are very limited [Wu, 05].

5 Conclusions

3-T_1AM and T_0AM have recently been identified as endogenous signaling molecules exhibiting remarkable *in-vivo* effects, such as rapid and profound hypothermia, hyperglycemia, reduction of the respiratory quotient, negative chronotropy and negative inotropy [Scanlan, 04; Braulke, 08]. So far, basically all fields of TAM biology are still open to research. It is not known if further representatives of TAMs apart from 3-T_1AM and T_0AM are present *in-vivo* and, if so, at which tissue concentrations and half lives. It remains elusive if the aforementioned *in-vivo* effects represent the physiological TAM actions or are merely pharmacological in nature. Moreover, the physiological receptor(s) of TAMs has (have) not been identified unequivocally. Further unsettled issues include the site, pathways and regulation of TAM biosynthesis, their transport in blood and across biological membranes as well as possible pathophysiological implications.

Considering the structural similarity of TAMs and THs, it has frequently been suggested that TAMs might be biosynthesized as metabolites of THs by decarboxylation of the β-alanine side chain. If the yet unidentified decarboxylating enzyme was converting only THs with higher iodine content, such as T_4, T_3s or T_2s, Dio isozymes would be directly required to complete the biosynthesis of 3-T_1AM and T_0AM by removing one to four iodine atoms. However, any role of Dio isozymes in TAM biosynthesis presupposes their ability to accept TAMs as substrates.

Given this possible new role of Dio isozymes in TAM biosynthesis, the present study aimed at analyzing the substrate specificities of the Dio isozymes towards TAMs. A particular strength of this study was its completeness since each Dio isozyme was analyzed with the complete panel of iodothyronamines. TAMs were subjected to *in-vitro* deiodination reactions catalyzed by isozyme specific Dio preparations, which had been validated extensively. To analyze the deiodination products, a novel liquid-liquid extraction protocol and an LC-MS/MS method were developed, which allowed for the unequivocal, simultaneous identification and quantification of all TAMs in the same sample. The extraction protocol did not

produce any interfering effects for the LC-MS/MS detection of the TAM analytes with the Dio reaction matrices. Furthermore, it yielded reproducible recovery rates, which were independent from the protein content and pH of the Dio reaction matrices. With the HPLC method developed herein, complete baseline separation of all except one pair of constitutional TAM isomers was achieved, which had not been accomplished by any other method, so far. Accordingly, the position of each iodine atom removed from a TAM or TH substrate in a Dio catalyzed reaction was backtracked unequivocally. Since the complete panel of THs as established Dio substrates was also included into the LC-MS/MS protocol, its performance in analyzing Dio catalyzed reactions was successfully compared to classical $^{125}I^-$ release assays. The position of the iodine atoms removed from TH substrates was always, without any exception, in line with the published Dio catalytic properties. Additionally, all TH substrates previously described were indeed converted by the respective Dio isozymes supporting the functionality of the Dio preparations and their suitability to study new Dio substrates.

The present study demonstrates that all Dio isozymes are capable of catalyzing TAM deiodination reactions with each Dio isozyme exhibiting a unique substrate specificity towards TAMs. The TAM deiodination reactions were analyzed using two independent analytical techniques. First of all, they were monitored directly by LC-MS/MS in the most comprehensive way ever reported for Dio catalyzed reactions. Both product formation, including the formation of sequential monodeiodination products, and substrate disappearance were monitored simultaneously and were both quantifiable. Secondly, the identified TAM substrates were functionally verified by inhibiting the Dio1 catalyzed phenolic ring deiodination of rT_3. Kinetic experiments demonstrated that with the Dio preparations and the reaction conditions used in this study, some TAMs represented better Dio substrates than the corresponding THs. Another interesting aspect of these data was that TAMs represent the only endogenous Dio substrates reported, so far, which possess a positively charged side chain at the tyrosyl ring.

Taken together, it is concluded that the present study supports a role for Dio isozymes in TAM biosynthesis. Thus, it also strengthens the hypothesis that TAMs are biosynthesized as TH metabolites. Moreover, by excluding a considerable number of TAM deiodination reactions, the biosynthetic pathways of the recently identified endogenous signaling molecules 3-T_1AM and T_0AM were confined. Hence, Dio isozymes do not only control the bioavailability of T_3 to classical nuclear thyroid hormone receptors but might also play an essential role in regulating the bioavailability of 3-T_1AM and T_0AM to their rapidly acting and putatively plasma membrane-bound receptors. Although their physiologic function remains elusive, 3-T_1AM and T_0AM have already revealed promising therapeutic potential since they represent the only endogenous compounds to induce hypothermia as a prophylactic or acute treatment of stroke and might thus be expected to cause fewer side effects than synthetic compounds [Doyle, 07]. Consequently, an improved knowledge of the biosynthetic pathways of 3-T_1AM and T_0AM will contribute to a better understanding of their pharmacokinetic properties and thus help to reduce side effects.

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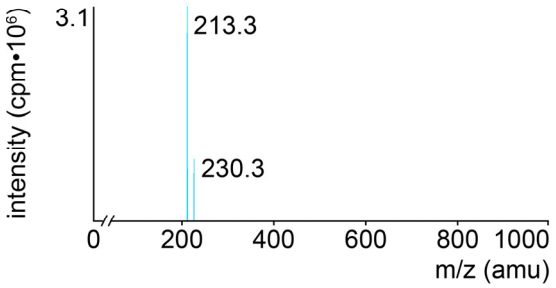
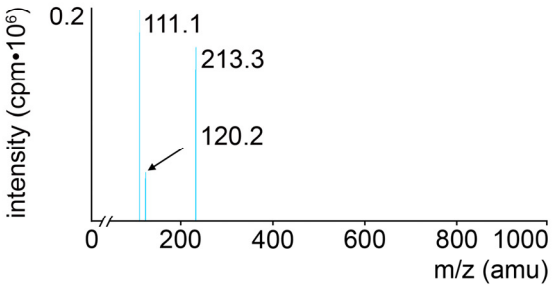
Supplementary figures and tables

Figure S28

A T_0AM

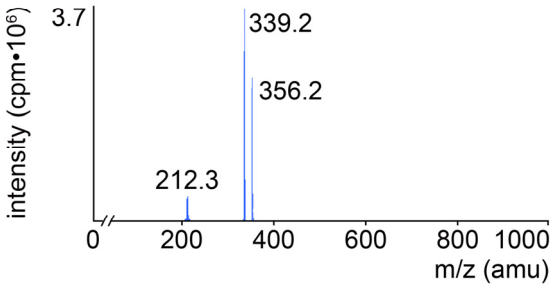


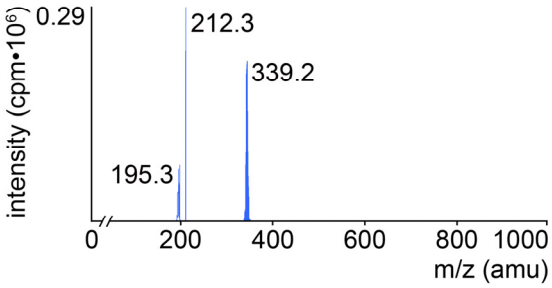


(m/z) (amu)	Interpretation
230.3	$(M+H)^{+}$ [a]
213.3	$(M+H-NH_3)^{+}$ [a]

(m/z) (amu)	Interpretation
120.2	$(M+H-1,4\text{-dihydroxybenzene})^{+}$
111.1	$(1,4\text{-dihydroxybenzene})^{+}$

B 3-T_1AM

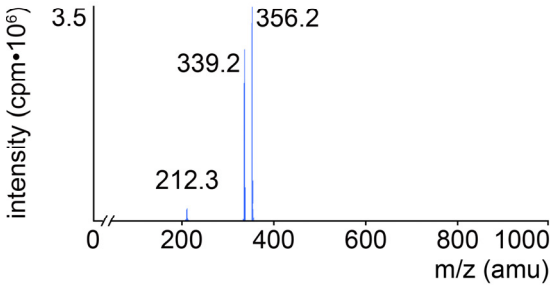


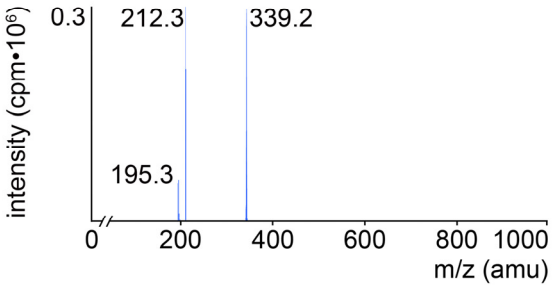


(m/z) (amu)	Interpretation
356.2	$(M+H)^{+}$ [a]
339.2	$(M+H-NH_3)^{+}$ [a]
212.3	$(M+H-NH_3-I)^{+}$ [a]

(m/z) (amu)	Interpretation
195.3	$(M+H-NH_3-I-OH)^{+}$

C 3'-T_1AM

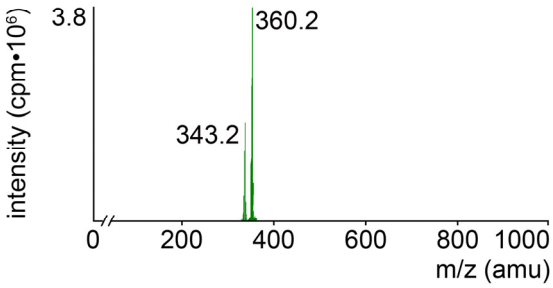


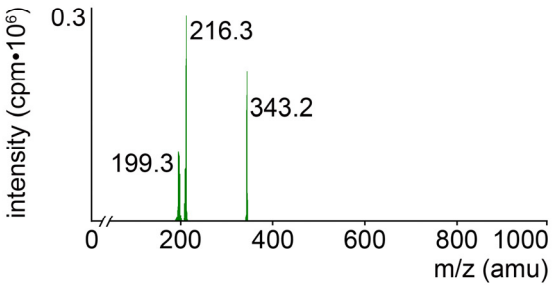


(m/z) (amu)	Interpretation
356.2	$(M+H)^+$
339.2	$(M+H-NH_3)^+$
212.3	$(M+H-NH_3-I)^+$

(m/z) (amu)	Interpretation
195.3	$(M+H-NH_3-I-OH)^+$

D 3-T_1AM-d4

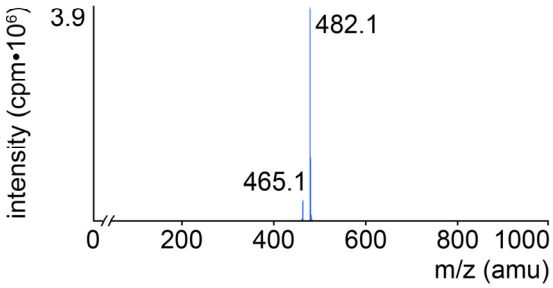


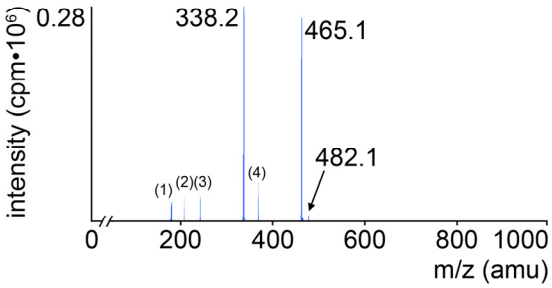


(m/z) (amu)	Interpretation
360.2	$(M+H)^+$
343.2	$(M+H-NH_3)^+$ or

(m/z) (amu)	Interpretation
216.3	$(M+H-NH_3-I)^+$
199.3	$(M+H-NH_3-I-OH)^+$

E 3,5-T_2AM

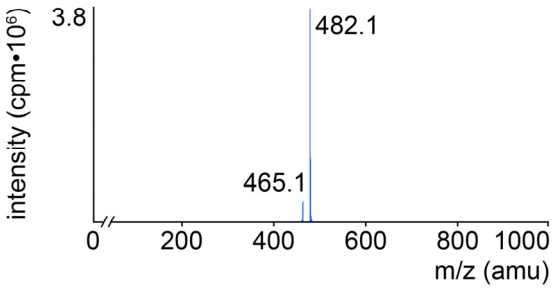


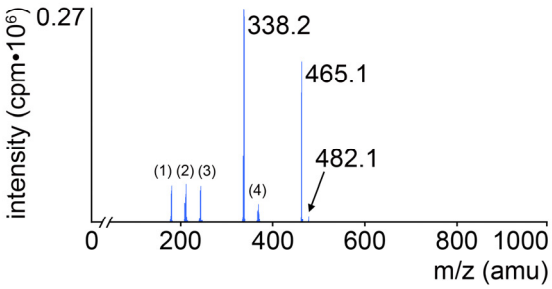


(m/z) (amu)	Interpretation
482.1	$(M+H)^+$ [a]
465.1	$(M+H-NH_3)^+$ [a]

(m/z) (amu)	Interpretation
372.0 (4)	$(M+H-$1,4-dihydroxybenzene$)^+$
338.2	$(M+H-NH_3-I)^+$
245.1 (3)	$(M+H-$ 1,4-dihydroxybenzene$-I)^+$ or $(M+H-NH_3-$ monohydroxybenzene$-I)^+$
211.3 (2)	$(M+H-NH_3-I-I)^+$
183.2 (1)	$(M+H-C_2H_7N-I-I)^+$

F 3,3'-T_2AM

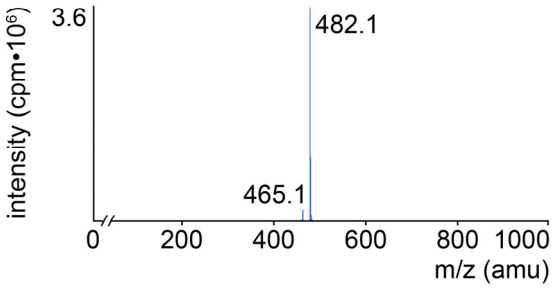


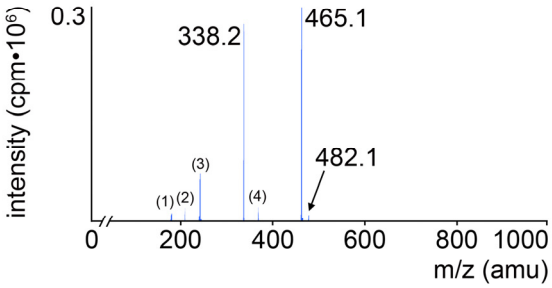


(m/z) (amu)	Interpretation
482.1	$(M+H)^+$ [a]
465.1	$(M+H-NH_3)^+$ [a]

(m/z) (amu)	Interpretation
372.0 (4)	$(M+H-$1,4-dihydroxybenzene$)^+$
338.2	$(M+H-NH_3-I)^+$
245.1 (3)	$(M+H-$ 1,4-dihydroxybenzene$-I)^+$ or $(M+H-NH_3-$ monohydroxybenzene$-I)^+$
211.3 (2)	$(M+H-NH_3-I-I)^+$
183.2 (1)	$(M+H-C_2H_7N-I-I)^+$

G 3',5'-T_2AM

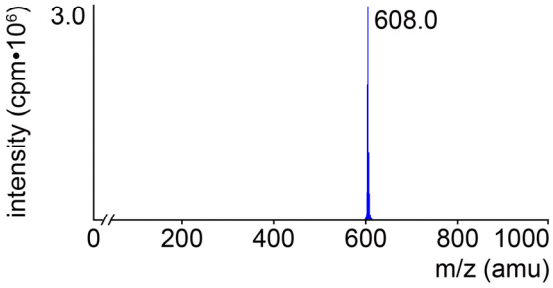


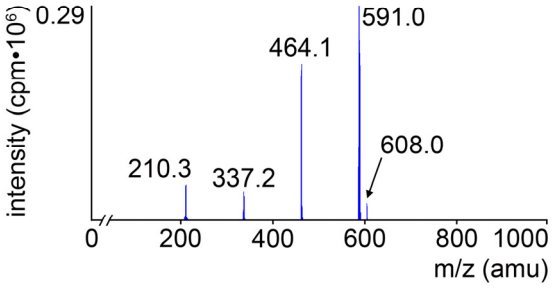


(m/z) (amu)	Interpretation
482.1	$(M+H)^+$ [a]
465.1	$(M+H-NH_3)^+$ [a]

(m/z) (amu)	Interpretation
372.0 [4]	$(M+H-$1,4-dihydroxybenzene$)^+$
338.2	$(M+H-NH_3-I)^+$
245.1 [3]	$(M+H-$ 1,4-dihydroxybenzene$-I)^+$ or $(M+H-NH_3-$ monohydroxybenzene$-I)^+$
211.3 [2]	$(M+H-NH_3-I-I)^+$
183.2 [1]	$(M+H-C_2H_7N-I-I)^+$

H T_3AM

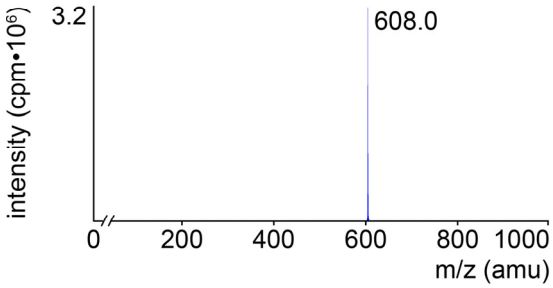


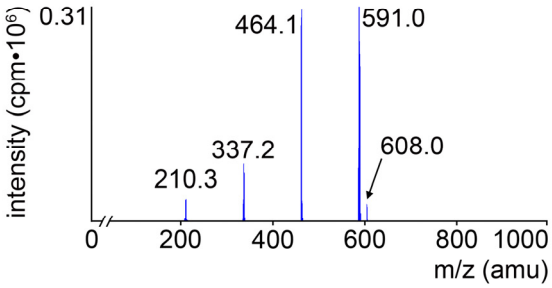


(m/z) (amu)	Interpretation
608.0	$(M+H)^+$

(m/z) (amu)	Interpretation
591.0	$(M+H-NH_3)^+$
464.1	$(M+H-NH_3-I)^+$
337.2	$(M+H-NH_3-I-I)^+$
210.3	$(M+H-NH_3-I-I-I)^+$

I rT_3AM

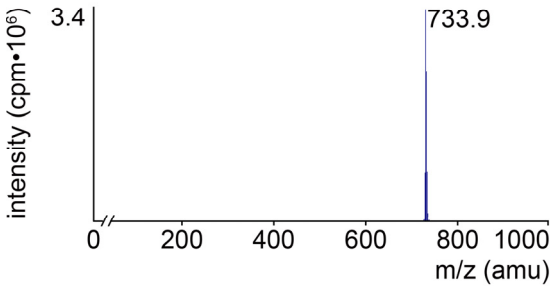


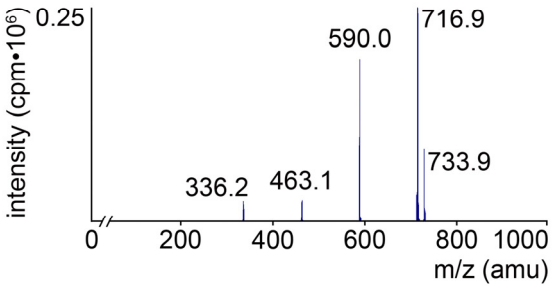


(m/z) (amu)	Interpretation
608.0	$(M+H)^+$

(m/z) (amu)	Interpretation
591.0	$(M+H-NH_3)^+$
464.1	$(M+H-NH_3-I)^+$
337.2	$(M+H-NH_3-I-I)^+$
210.3	$(M+H-NH_3-I-I-I)^+$

J T_4AM

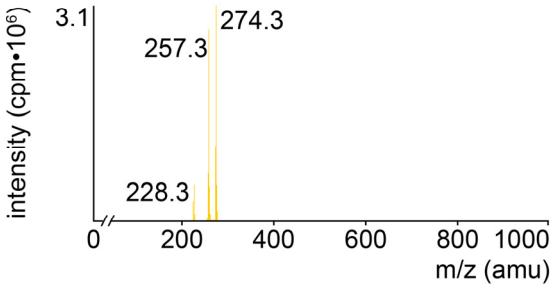


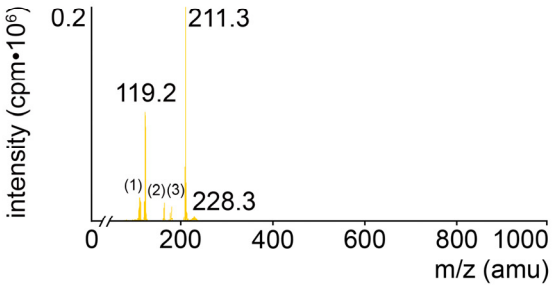


(m/z) (amu)	Interpretation
733.9	$(M+H)^+$

(m/z) (amu)	Interpretation
716.9	$(M+H-NH_3)^+$
590.0	$(M+H-NH_3-I)^+$
463.1	$(M+H-NH_3-I-I)^+$
336.2	$(M+H-NH_3-I-I-I)^+$

K T_0

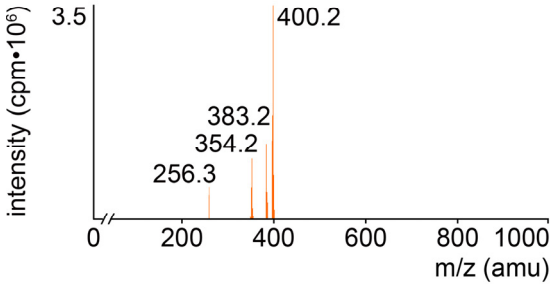


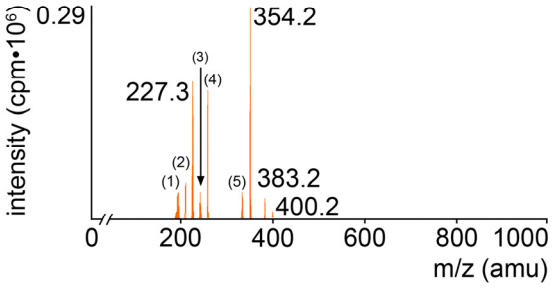


(m/z) (amu)	Interpretation
274.3	$(M+H)^+$
257.3	$(M+H-NH_3)^+$
228.3	$(M+H-HCOOH)^+$

(m/z) (amu)	Interpretation
211.3	$(M+H-NH_3-HCOOH)^+$
182.2 [3]	$(tyrosine)^+$
164.3 [2]	$(M+H-1,4\text{-dihydroxybenzene})^+$
119.2	$(tyrosine-NH_3-HCOOH)^+$
111.1 [1]	$(1,4\text{-dihydroxybenzene})^+$

L 3-T_1

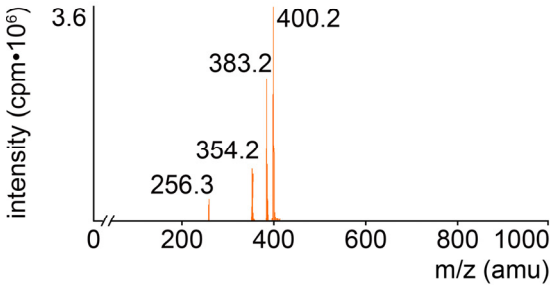


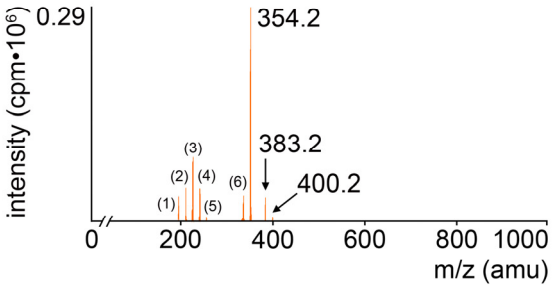


(m/z) (amu)	Interpretation
400.2	$(M+H)^{+\,b}$
383.2	$(M+H-NH_3)^{+\,b}$
354.2	$(M+H-HCOOH)^{+\,b}$
256.3	$(M+H-NH_3-I)^{+\,b}$ or $(M+H-OH-I)^{b}$

(m/z) (amu)	Interpretation
337.2 [5]	$(M+H-NH_3-HCOOH)^{+\,b}$
256.3 [4]	$(M+H-NH_3-I)^{+\,b}$ or $(M+H-OH-I)^{b}$
244.2 [3]	$(M+H-HCOOH-1,4\text{-dihydroxybenzene})^+$
227.3	$(M+H-HCOOH-I)^+$
210.3 [2]	$(M+H-NH_3-HCOOH-I)^+$
199.2 [1]	$(M+H-CH_4O_2N-I)^+$

M 3'-T_1

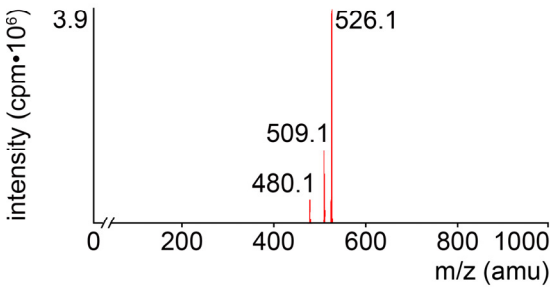


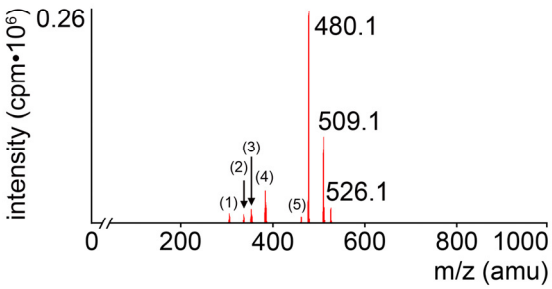


(m/z) (amu)	Interpretation
400.2	$(M+H)^{+}$ [b]
383.2	$(M+H-NH_3)^{+}$ [b]
354.2	$(M+H-HCOOH)^{+}$ [b]
256.3	$(M+H-NH_3-I)^{+}$ [b] or $(M+H-OH-I)$ [b]

(m/z) (amu)	Interpretation
337.2 (6)	$(M+H-NH_3-HCOOH)^{+}$ [b]
256.3 (5)	$(M+H-NH_3-I)^{+}$ [b] or $(M+H-OH-I)$ [b]
244.2 (4)	(M+H-HCOOH-1,4-dihydroxybenzene)$^{+}$
227.3 (3)	$(M+H-HCOOH-I)^{+}$
210.3 (2)	$(M+H-NH_3-HCOOH-I)^{+}$
199.2 (1)	$(M+H-CH_4O_2N-I)^{+}$

N 3,5-T_2

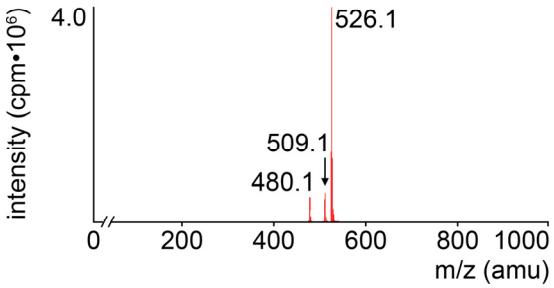


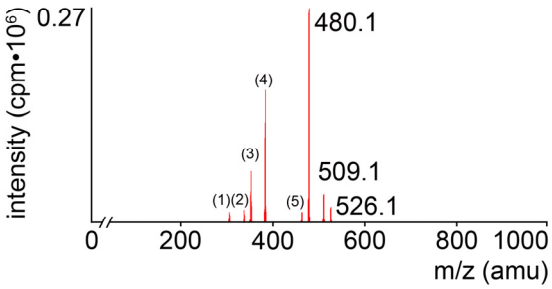


(m/z) (amu)	Interpretation
526.1	$(M+H)^{+}$ [c]
509.1	$(M+H-NH_3)^{+}$ [c]
480.1	$(M+H-HCOOH)^{+}$ [c]

(m/z) (amu)	Interpretation
463.1 (5)	$(M+H-NH_3-HCOOH)^{+}$ [c]
382.2 (4)	$(M+H-NH_3-I)^{+}$ [c] or $(M+H-OH-I)$ [c]
353.2 (3)	$(M+H-NH_3-I-I)^{+}$
336.2 (2)	$(M+H-NH_3-HCOOH-I)$
310.2 (1)	$(M+H-NH_3-\beta\text{-alanine}-I)^{+}$

O 3,3'-T_2

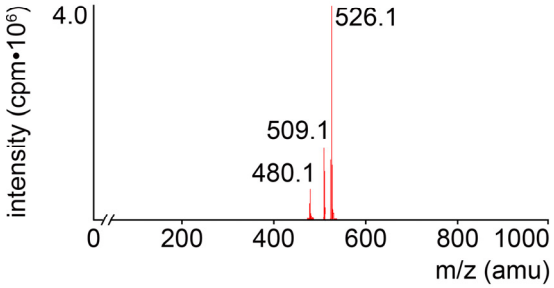


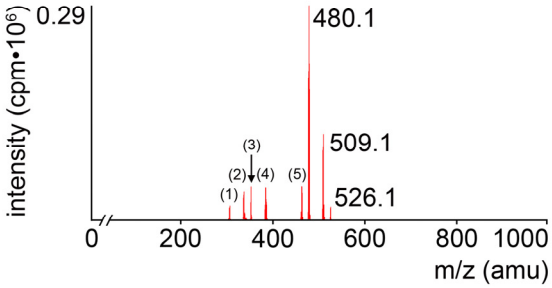


(m/z) (amu)	Interpretation
526.1	$(M+H)^{+}$ [c]
509.1	$(M+H-NH_3)^{+}$ [c]
480.1	$(M+H-HCOOH)^{+}$ [c]

(m/z) (amu)	Interpretation
463.1 [(5)]	$(M+H-NH_3-HCOOH)^{+}$ [c]
382.2 [(4)]	$(M+H-NH_3-I)^{+}$ [c] or $(M+H-OH-I)$ [c]
353.2 [(3)]	$(M+H-NH_3-I-I)^{+}$
336.2 [(2)]	$(M+H-NH_3-HCOOH-I)$
310.2 [(1)]	$(M+H-NH_3-\beta\text{-alanine}-I)^{+}$

P 3',5'-T_2

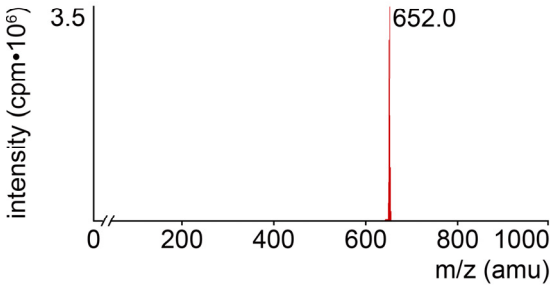


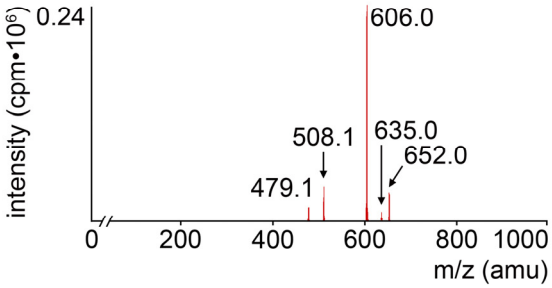


(m/z) (amu)	Interpretation
526.1	$(M+H)^{+}$ [c]
509.1	$(M+H-NH_3)^{+}$ [c]
480.1	$(M+H-HCOOH)^{+}$ [c]

(m/z) (amu)	Interpretation
463.1 [(5)]	$(M+H-NH_3-HCOOH)^{+}$ [c]
382.2 [(4)]	$(M+H-NH_3-I)^{+}$ [c] or $(M+H-OH-I)$ [c]
353.2 [(3)]	$(M+H-NH_3-I-I)^{+}$
336.2 [(2)]	$(M+H-NH_3-HCOOH-I)$
310.2 [(1)]	$(M+H-NH_3-\beta\text{-alanine}-I)^{+}$

Q T_3

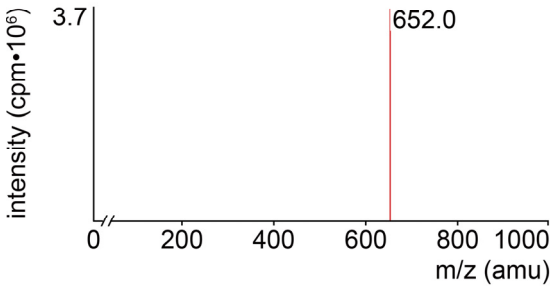


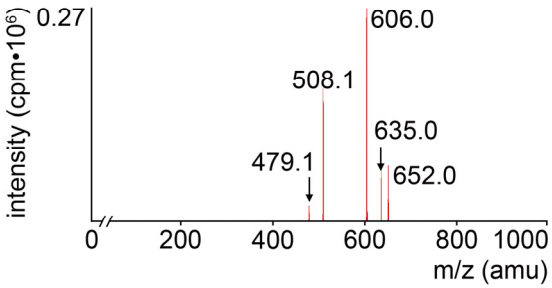


(m/z) (amu)	Interpretation
652.0 [d, e]	$(M+H)^+$

(m/z) (amu)	Interpretation
635.0	$(M+H-NH_3)^+$
606.0	$(M+H-HCOOH)^+$ [d, e]
508.1	$(M+H-NH_3-I)^+$
479.1	$(M+H-HCOOH-I)^+$

R rT_3

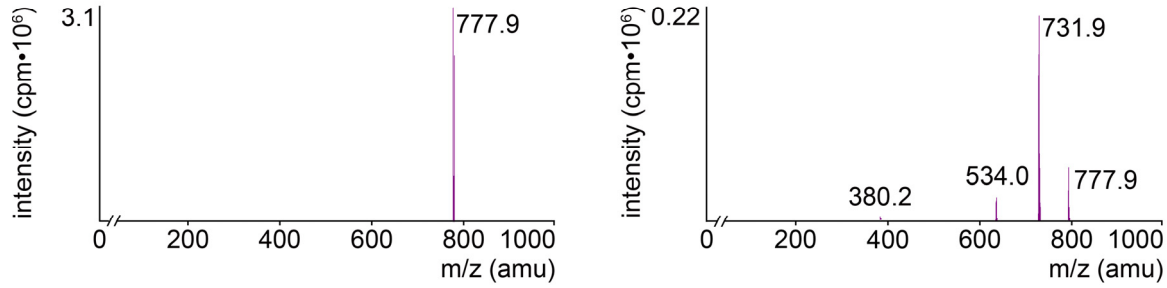




(m/z) (amu)	Interpretation
652.0	$(M+H)^+$

(m/z) (amu)	Interpretation
635.0	$(M+H-NH_3)^+$
606.0	$(M+H-HCOOH)^+$
508.1	$(M+H-NH_3-I)^+$
479.1	$(M+H-HCOOH-I)^+$

S T_4



(m/z) (amu)	Interpretation
777.9 [f, g]	$(M+H)^+$

(m/z) (amu)	Interpretation
731.9 [f]	$(M+H-HCOOH)^+$
634.0	$(M+H-NH_3-I)^+$
380.2	$(M+H-NH_3-I-I-I)^+$

Figure S28: Representative parent ion mass spectra (**left panel**) and product ion tandem mass spectra (**right panel**) of 10 µg/ml analyte working solutions. The parent ion mass spectra show the optimized detection of the parent ions (largest mass in each spectrum) in the first quadrupole. The product ion tandem mass spectra outline the optimized detection of the most intensive product ion, namely $(M+H-NH_3)^+$ in the case of TAMs (**A - J**) and $(M+H-HCOOH)^+$ in the case of THs (**K - S**).

[a] [Scanlan, 04]

[b] [Zhang, 06b]

[c] [Zhang, 06a]

[d] [Thienpont, 99]

[e] [Tai, 04]

[f] [De Brabandere, 98]

[g] [Tai, 02]

Table S20: Exemplary matrix effects obtained for Dio reactions which contained HepG2 lysates and were devoid of PTU.

Analyte	**Analyte concentration in the Dio reactions**		
	10 nM	**500 nM**	**7.5 µM**
T_0AM	101 ± 4.8	104 ± 5.9	103 ± 4.2
3-T_1AM	96 ± 3.7	102 ± 2.7	96 ± 4.1
3-T_1AM-d4	99 ± 4.4	101 ± 4.1	96 ± 5.2
3'-T_1AM	103 ± 2.4	99 ± 2.1	98 ± 2.0
3,5-T_2AM	102 ± 4.9	103 ± 4.1	104 ± 4.8
3,3'-T_2AM	100 ± 1.7	101 ± 1.3	99 ± 2.4
3',5'-T_2AM	101 ± 4.3	99 ± 2.9	98 ± 4.5
T_3AM	99 ± 4.9	104 ± 5.5	99 ± 5.4
rT_3AM	97 ± 3.6	100 ± 3.3	101 ± 2.8
T_4AM	100 ± 6.7	96 ± 4.9	98 ± 5.8
T_0	103 ± 7.0	97 ± 6.3	98 ± 6.6
3-T_1	98 ± 1.2	104 ± 2.5	96 ± 1.9
3'-T_1	107 ± 2.3	98 ± 1.6	103 ± 0.9
3,5-T_2	96 ± 4.8	96 ± 3.9	100 ± 5.1
3,3'-T_2	104 ± 5.0	101 ± 4.6	96 ± 4.8
3',5'-T_2	99 ± 2.4	98 ± 3.1	104 ± 3.0
T_3	98 ± 4.7	99 ± 5.4	98 ± 4.2
rT_3	103 ± 3.1	104 ± 4.0	109 ± 2.7
T_4	103 ± 5.2	96 ± 4.1	99 ± 4.6

Table S21: Exemplary process efficiencies obtained for Dio reactions which contained HepG2 lysates and were devoid of PTU.

Analyte	**Analyte concentration in the Dio reactions**		
	10 nM	**500 nM**	**7.5 µM**
T_0AM	59 ± 5.1	62.4 ± 5.0	57 ± 4.9
3-T_1AM	79 ± 4.2	85 ± 3.5	87 ± 3.9
3-T_1AM-d4	82 ± 5.2	91 ± 5.7	79 ± 4.9
3'-T_1AM	89 ± 3.8	86 ± 5.2	81 ± 5.6
3,5-T_2AM	82 ± 1.7	85 ± 3.6	87 ± 1.5
3,3'-T_2AM	81 ± 2.6	83 ± 1.8	79 ± 2.5
3',5'-T_2AM	83 ± 4.0	80 ± 3.7	77 ± 4.1
T_3AM	60 ± 4.7	63 ± 4.6	61 ± 3.9
rT_3AM	60 ± 4.6	64 ± 3.2	65 ± 3.8
T_4AM	50 ± 7.0	49 ± 6.8	50 ± 6.1
T_0	48 ± 5.4	46 ± 5.1	48 ± 4.9
3-T_1	82 ± 4.1	85 ± 3.2	79 ± 4.7
3'-T_1	93 ± 1.6	89 ± 1.9	90 ± 2.8
3,5-T_2	80 ± 2.0	80 ± 3.3	84 ± 2.1
3,3'-T_2	90 ± 3.1	88 ± 3.6	96 ± 2.4
3',5'-T_2	88 ± 3.9	88 ± 3.4	92 ± 4.4
T_3	69 ± 6.0	70 ± 5.7	68 ± 6.2
rT_3	74 ± 5.9	75 ± 5.6	78 ± 5.9
T_4	56 ± 3.6	56 ± 4.0	53 ± 5.1

Publications

Peer-reviewed journals

Piehl S., Heberer T., Balizs G., Köhrle J. (2008): Development of a validated LC-MS/MS method for the distinction of thyronine and thyronamine constitutional isomers and for the identification of new deiodinase substrates. (in preparation)

Piehl S., Heberer T., Balizs G., Scanlan T.S., Smits R., Koksch B., Köhrle J. (2008): Thyronamines are isozyme specific substrates of deiodinases. (accepted)

Oral presentations

Piehl, S., Köhrle J. (2007): Novel insights into thyronamine biosynthesis. 23rd Workshop on Experimental Thyroid Research (AESF), Lübeck, Germany, December 6 - 8 2007

Piehl, S., Köhrle J. (2006): Possible roles of deiodinase type 1 and 2 in thyronamine biosynthesis. 22nd Workshop on Experimental Thyroid Research (AESF), Berlin, Germany, December 7 - 9 2006

Piehl, S., Köhrle J. (2006): Possible roles of deiodinase type 1 in thyronamine biosynthesis. 8th Annual Conference of the German Society of Endocrinology -Young Active Research (YAR) in Würzburg, Germany, October 6 - 8, 2006

Piehl, S., Köhrle J. (2005): Biosynthesis of thyronamines - Concept for a PhD thesis. 21st Workshop on Experimental Thyroid Research (AESF), Halle, Germany, December 1 - 3 2005

Poster presentations

Piehl S., Balizs G., Heberer T., Scanlan T.S., Smits R., Koksch B., Köhrle J. (2008): Thyronamines are isozyme specific substrates of deiodinases. 10th European Congress of Endocrinology (ECE), Berlin, Germany, May 3 - 7 2008

Piehl, S., Heberer T., Balizs G., Köhrle J. (2007): Deiodinase type 1 catalyzes specific thyronamine deiodination reactions. Bregenz Summer School on Endocrinology, Bregenz, Austria, July 22 - 26 2007

Piehl, S., Heberer T., Balizs G., Köhrle J. (2007): The role of deiodinase type 1 in thyronamine biosynthesis. 9th European Congress of Endocrinology (ECE), Budapest, Hungary, April 28 - May 2 2007

Piehl, S., Heberer T., Balizs G., Köhrle J. (2007): A detection method for thyronamines based on liquid-liquid extraction and LC-MS/MS detection. 51st Symposium of the German Society of Endocrinology (DGE), Salzburg, Austria, March 7 - 10 2007

Piehl, S., Heberer T., Balizs G., Köhrle J. (2006): An LC-MS/MS based method for the detection of thyronamines. Bregenz Summer School on Endocrinology, Bregenz, Austria, July 30 - August 3 2006

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Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit mit dem Titel „The roles of deiodinases in thyronamine biology“ selbständig und nur unter Verwendung der angegebenen Literatur und Hilfsmittel angefertigt habe. Desweiteren erkläre ich meine Kenntnisnahme der dem angestrebten Verfahren zugrunde liegenden Promotionsverordnung. Ich habe mich anderwärts nicht um einen Doktorgrad beworben und bin nicht im Besitz eines entsprechenden Doktorgrades.

Berlin, den 03. Juli 2008

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Susanne Piehl